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TESTOSTERONE AND TISSUE RESPIRATION OF THE CASTRATE MALE RAT WITH A POSSIBLE TEST FOR MYOTROPHIC ACTIVITY[1]

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ALTHOUGH the general metabolic effects of gonadal steroids have long been recognized, the mechanisms by which they exert these effects are as yet poorly understood. Jensen and Tenenbaum, in their 1944 review of endocrine enzyme relationships, noted that there were no reports on the relations of these hormones to specific enzyme systems *in vitro*. Since that time Kochakian has extensively studied the effects of castration and of various steroids upon the acid and alkaline phosphatases and arginases in several tissues (Kochakian, 1947). On the basis of these studies Kochakian (1946) has suggested that kidney arginase may be associated with the protein-anabolic properties of the steroids. Clark, Kochakian, and Fox (1943) have found that testosterone increases the d-amino acid oxidase of the mouse kidney. Kochakian (1943) has also showed a similar increase in the activity of this enzyme in liver. Everett and Sawyer (1946) have found that testosterone lowers the serum content of non-specific cholinesterase in the rat. McShan, Meyer, and Erway (1947) have studied the effect of various soluble steroids upon succinoxidase systems. They found no effect from the sodium sulfate conjugates of the androgens androsterone and isodehydro-androsterone. Erway, Meyer, and McShan (1947) have likewise studied malic dehydrogenase, which was markedly depressed by several synthetic estrogens, but was not affected by sodium androsterone sulfate.

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In 1947 Gordan and Elliott found that testosterone added *in vitro* depresses the aerobic respiration of rat brain in a glucose substrate, presumably at the dehydrogenase level. In an attempt to determine the physiological significance of this phenomenon Eisenberg, Gordan, and Elliott (1949) studied the effect of castration upon glucose oxidation by rat brain. They found that castration produced a marked increase in the rate of glucose utilization. Administration of testosterone *in vivo* prevented this increase. It was concluded that testosterone may act as a "metabolic brake" upon the oxidation of glucose by rat brain. It was further found that the brain of the castrated male rat is less sensitive to the oxidation-inhibiting properties of testosterone than that of the normal or testosterone-treated castrate. These observations have been extended to other tissues and are reported here. Studies have now been made upon the brain, liver, diaphragm, and another striated muscle, the levator ani.

It will be recalled that Wainman and Shipounoff (1941) found a stimulating effect of testosterone upon the perineal complex (penis and its bulb, the glands of the bulb, bulbocavernosus, ischiocavernosus, and levator ani muscles) in the rat. Their experiments clearly demonstrated an atrophy of the perineal musculature after castration of the male rat and an enlargement of this complex in the male and female rat upon administration of testosterone. Histological examination showed this effect to be related to a decrease or increase in width of the muscular fibers. Unfortunately, the complex was weighed as a whole and individual muscle weights were not reported. Papanicolaou and Falk (1938) have reported a visible decrease in the bulk of the temporal muscle as well as other muscles of the male guinea pig following castration and this effect was likewise prevented by the administration of testosterone. The present experiment afforded the opportunity to study the effect of castration and testosterone upon muscle weight directly. In preliminary experiments it has been found that the levator ani is technically the most suitable of the group for study of this myotrophic action.

METHOD

Tissues were studied from three groups of male rats: 1) a group castrated at 30 days of age; 2) a second group which was castrated at the same age and injected subcutaneously with one milligram of testosterone propionate[2] in sesame oil daily during the following 30 days; 3) and a third group of 60 day old normal males. Rats of the Long-Evans and Slonaker-Wistar strains were used; since data from the two strains were identical, the results were combined. All animals were decapitated at 60 days of age.

The levator ani was removed with the entire perineal complex by splitting the symphysis pubis, severing the attachments of the ischiocavernosus, dividing the urethra and rectum superiorly and dissecting the complex from

[2] The testosterone propionate (Perandren) was generously contributed by Ciba Pharmaceutical Products, Inc., Summit, New Jersey.

the rectum. All tissues were placed in a cold box (4° C.) where the levator ani was cut from the bulbocavernosus. Because of the thickness of the levator ani in the normal and testosterone-treated castrate rats, this muscle was split in half longitudinally. In the castrate rats splitting was not necessary because of the marked atrophy of the levator ani. The thinner anterior and lateral portions of the diaphragm were divided into 40 to 50 mg. segments. Liver slices of 0.5 mm. thickness were prepared with a Martin slicer (1942).

The tissues were weighed on a torsion balance and placed in a Warburg flask containing modified Krebs-Ringer phosphate buffer (Extracellular Ringer's, or ER). Liver and diaphragm were divided into two groups: one in ER without added substrate and the other in ER to which 0.2 per cent glucose had been added (Extracellular Glucose-Ringer's, or EGR). In addition, brain studies in ER containing 0.2 per cent sodium pyruvate were made in order to determine whether the primary site of the *in vitro* inhibition is in the fermentative or oxidative phase of glucose utilization (Field, 1947). When testosterone was added *in vitro* it was placed in the side arm as 0.2 ml. of an aqueous suspension containing 2 mg. of finely powdered testosterone.[3] This dose was employed *in vitro* since it was found to result in the greatest degree of inhibition per mg., 4 and 8 mg. producing little added effect, lesser doses producing a relatively smaller response. Administration of such large amounts of steroid was necessitated by the relative insolubility of testosterone in an aqueous medium. By exposing the tissue to the suspension, penetration of a sufficient amount of the agent to produce an inhibition of oxidative metabolism was effected. In order to eliminate a non-specific action, several carcinogens (3 methyl-cholanthrene, 1,2-benzanthracene, 1,2,5,6-dibenzanthracene, and 3,4-benzpyrene)[4] with phenanthrene nuclei were also added *in vitro*. Other steroidal agents in the same amount have been included as controls in an earlier report (Gordan and Elliott).

The total volume of the flasks was made up to 2 ml. The flasks were placed in a bath at 37.2° C. and readings were taken every 15 minutes for 180 minutes. When agents were added *in vitro* they were tipped in from the side arm at 90 minutes. The gas phase was oxygen, the CO_2 being absorbed by 0.1 ml. of 10 per cent KOH on filter paper in the center well. QO_2 is reported as microliters (μl) of oxygen used per mg. of dry tissue per hour.

EXPERIMENTAL DATA

Examination of the data presented in Table 1 shows that the oxygen uptake of the tissues studied was inhibited by testosterone *in vitro* both in ER and EGR. It may be further noted that the initial respiratory rate of these tissues was not elevated following castration in contrast to the increase previously reported for brain. The most striking observation was the reduced ability of testosterone to inhibit the respiration of castrate rat tissues, an effect also found with brain. While the per cent of inhibition of the levator ani from the castrate, normal, and treated-castrate rats is the same, the data suggest a de-

[3] We are indebted to Dr. E. Oppenheimer, Ciba Pharmaceutical Products, Inc., Summit, New Jersey, for crystalline testosterone used in this experiment.

[4] Kindly furnished by Dr. M. B. Shimkin, Laboratory of Experimental Oncology, University of California Medical School, San Francisco, California.

crease in sensitivity of the muscle from castrate rats as was found with the other tissues. Inasmuch as the initial QO_2's of levator ani from all three groups of animals were the same, the identical inhibition of the respiration of this muscle by testosterone *in vitro* is probably explainable on the basis that the QO_2 of the very thin muscle from the cas-

TABLE 1. OXYGEN UPTAKE OF LIVER, DIAPHRAGM AND LEVATOR ANI OF NORMAL, CASTRATE, AND TESTOSTERONE-TREATED CASTRATE RATS

Tissue	QO_2 during first 90′	QO_2 at end of 180′	QO_2 at end of 180′—testosterone added at 90′	% of inhibition
Liver				
Normal				
EGR	6.5 ±0.2 (32)*	5.3 ±0.7 (8)	2.7 ±0.1 (24)	49
ER	6.1 ±0.2 (28)	4.7 ±0.3 (8)	2.2 ±0.1 (20)	53
Castrate				
EGR	5.2 ±0.2 (34)	3.8 ±0.6 (10)	3.0 ±0.2 (24)	21
ER	5.7 ±0.1 (28)	4.1 ±0.2 (10)	3.0 ±0.1 (18)	27
Castrate-treated				
EGR	4.5 ±0.3 (33)	3.4 ±0.5 (12)	1.9 ±0.2 (21)	44
ER	4.8 ±0.2 (34)	3.7 ±0.4 (12)	1.7 ±0.1 (23)	54
Diaphragm				
Normal				
EGR	6.3 ±0.1 (30)	5.8 ±0.5 (6)	3.2 ±0.1 (24)	45
ER	6.3 ±0.1 (28)	5.7 ±0.4 (6)	3.1 ±0.1 (22)	46
Castrate				
EGR	5.9 ±0.1 (35)	5.6 ±0.1 (12)	3.8 ±0.1 (23)	32
ER	6.1 ±0.1 (30)	5.2 ±0.2 (8)	3.7 ±0.1 (22)	29
Castrate-treated				
EGR	5.9 ±0.1 (29)	5.4 ±0.4 (8)	3.0 ±0.1 (21)	44
ER	5.5 ±0.1 (30)	5.0 ±0.3 (8)	2.7 ±0.1 (22)	46
Levator Ani				
Normal				
EGR	3.5 ±0.4 (23)	2.5 ±0.5 (5)	1.4 ±0.1 (18)	44
Castrate				
EGR	3.4 ±0.2 (14)	3.7 ±0.3 (5)	2.1 ±0.2 (9)	43
Castrate-treated				
EGR	3.2 ±0.1 (23)	2.0 ±0.1 (7)	1.1 ±0.1 (16)	45

* Figures are the mean ± standard deviation of the mean; figures in parentheses indicate the number of vessels.

trate rats was better maintained over the three hour experimental period than that of the thicker muscles from normal and castrate-treated rats (see Table 1). This question will be studied in future experiments.

The weight changes are listed in Table 2. A marked reduction in weight of the levator ani is evident in the castrate rat. Treatment with testosterone not only prevented this atrophy but produced an increase in weight beyond the normal range. Wet : dry ratios (Table 2) of liver and diaphragm were unchanged by castration but lowered in

the case of levator ani. The maintenance of protein plus protein bound water by testosterone is adequate to explain the increased wet: dry ratio of the muscle of the treated castrate. The slight discrepancy can be explained by the persistence of connective tissue elements.

Studies on brain not previously reported include the observation that testosterone inhibits pyruvate oxidation by brain cell suspensions to the same degree as it inhibits glucose oxidation. This indicates that the primary inhibitory action of testosterone is not in the fermentative phase of glucose utilization. In addition, four phenanthrene carcinogens added to brain cell suspension had no effect on oxygen uptake whatsoever.

TABLE 2. EFFECT OF CASTRATION AND OF TESTOSTERONE UPON BODY AND ORGAN WEIGHTS

Group	No.	Body Weight in grams	Levator ani			Diaphragm		Liver
			Wet wgt. in mg.	W:D*	% of normal wet wgt.	Wet wgt. in mg.	W:D*	W:D*
Normal	12	182 ±8.1‡	81 ±6	4.9	100	304 ±11	4.5	3.4
Castrate	16	161 ±4.1	37 ±3	4.2	46	267 ±12	4.3	3.2
Castrate-treated	13	166 ±8.3	147 ±9	5.3	181	393 ±19	4.2	3.2

* W:D is the ratio of wet weight to dry weight.
‡ Figures are the mean ±standard deviation of the mean.

DISCUSSION

The data presented above and in previous reports show that suspensions of testosterone inhibit the oxygen uptake of rat brain, liver and striated muscle preparations as determined by the direct method of Warburg. The effect is specific and not due to the steroidal nature of the molecule since several other phenanthrenes in the same amounts and under the same conditions do not inhibit the oxygen uptake of brain homogenates. In addition, the inhibition reported for other steroids differs quantitatively from testosterone and from compound to compound. Studies on brain preparations to elucidate the site of action indicate: 1) the cytochromes are not involved since amounts of testosterone which inhibit glucose oxidation do not interfere with the oxidation of succinate; 2) inhibition is not primarily in the fermentative phase of glucose utilization since pyruvate oxidation is inhibited as effectively as glucose oxidation, and 3) the primary site of inhibition is presumably at the dehydrogenase level of the main line of biological oxidation since inhibition of glucose oxidation is not reversed by methylene blue.

Although these findings cannot be directly transferred to the in-

tact animal because the amount of testosterone which penetrated the tissue is unknown, other evidence suggests that testosterone may be implicated in *in vivo* oxidative processes. First, brain from castrated rats has a greater rate of oxygen consumption than brain from normal rats or testosterone-treated castrates, and second, tissue from castrated animals is less sensitive to the action of testosterone suspensions *in vitro*. The hypothetical oxidative system in which testosterone is thought to play a part seems to "atrophy" after castration, such "atrophy" being prevented by administration of testosterone to the castrate animal. It is of interest that Davis, Meyer, and McShan (1949) report decreased succinic dehydrogenase and cytochrome oxidase activity in the seminal vesicle and prostate of the rat following castration and that the administration of androgen maintains these factors at the normal level. This correlates directly with the lower QO_2 of prostate incident to castration reported by Guzman Barron and Huggins.

The elevation of brain QO_2 following castration might be related to an increase in brain hexokinase activity as a result of castration such as is reported following hypophysectomy (Reiss and Rees, 1947) but this alone will not explain all the findings. Integration of the information obtained from the four tissues studied makes it seem likely that some as yet unidentified enzyme system degenerates in the absence of testosterone. In brain respiring in EGR this is manifested as the removal of a metabolic "brake" as well as by a decreased sensitivity to testosterone *in vitro*. In liver and striated muscle, which can respire equally well in ER or EGR, the decreased sensitivity to testosterone persists. The lack of rise in QO_2 of liver and striated muscle following castration, in contrast to brain, would seem to be linked to the ability of these tissues to use endogenous substrates.

The changes in weight of the levator ani confirm the work of Wainman and Shipounoff on the relation of testosterone to the striated perineal musculature. Since this muscle is more responsive to castration and testosterone than other striated muscles in the rat, it is possible that it may be used as an index of the myotrophic effect of the steroid hormones. Studies on the specificity and reliability of this effect as an indicator of general protein anabolic activity are now in progress.

SUMMARY

The oxygen uptake of liver, diaphragm, and levator ani is not increased by castration as it is in the case of brain.

Castration renders diaphragm and liver relatively insensitive to the oxidation-inhibiting property of testosterone regardless of the presence or absence of glucose. A normal degree of reactivity is preserved in the castrate rat treated with testosterone.

Castration produces marked atrophy of the levator ani muscle of the male rat. Testosterone prevents this atrophy and, in the doses

used, produces an hypertrophy. The possible use of this muscle as an indicator of myotrophic activity is discussed.

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THE MECHANISM OF ACTION OF *LITHOSPERMUM RUDERALE*

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THE ESTABLISHMENT of the activity of *Lithospermum ruderale* as a substance which produces pronounced suppression of the female reproductive cycle in mice (Cranston, 1945) has resulted in several inquiries into the exact mechanism whereby it produces its effect (Drasher and Zahl, 1946; Zahl, 1948). All of these investigations have led to the conclusion that *Lithospermum* acts by a direct suppression of the power of the anterior pituitary to produce adequate amounts of gonadotrophins. More recently, Cranston and Robinson (1949) have shown that the pituitaries of female mice which have received *Lithospermum* for a week or more show a marked reduction of gonadotrophin potency as was demonstrated by assay on immature female mice. Somewhat similar work has been in progress in this laboratory and, therefore, it seems advisable to confirm the experiments of Cranston and Robinson at this time as well as to bring forth evidence for the possible differential effect of *Lithospermum* upon the two major gonadotrophic secretions of the pituitary, the follicle-stimulating hormone (FSH) and the luteinizing hormone (LH). Such a differential effect would be of considerable significance in the proposed clinical use of this drug.

Because of the possibility of other factors (dietary or endocrine) producing inhibition of the pituitary, it was deemed necessary to investigate these avenues more completely than had been done previously before concluding that *Lithospermum* exerts its effect directly upon the pituitary.

Previous work has adequately eliminated the possibility of a deficiency of vitamins A, C, D, or E being indirectly responsible for the anterior pituitary inhibition which attends *Lithospermum* administration. Evans and Simpson (1930) have reported however that vitamin B deficiency results in a decrease of pituitary gonadotrophin potency in the rat. Despite the fact that animals on a *Lithospermum* diet exhibit none of the gross symptoms of such a deficiency, it is conceivable the

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pituitary might have a threshold so low that a borderline deficiency of this vitamin complex could produce inhibition of secretory activity. The members of the B complex are intimately associated with the cellular enzyme systems, and since such enzyme systems must be linked closely with the chain of events leading to the production of the secretory products of the pituitary cells, the effect of vitamin B supplementation was investigated, the results of which are reported below.

Chu (1944) has reported that the pituitaries of thyroidectomized rabbits are free of LH but contain increased amounts of FSH. The possibility of a similar differential effect of *Lithospermum* discussed below, made it advisable to rule out thyroid implication by more precise methods than had been used to date. The determination of basal metabolic rate by Cranston (1945) and histological examination by Zahl (1948) may be considered insufficiently sensitive measures of thyroid activity. Accordingly, histometric analysis of the epithelium of the thyroid follicle was undertaken.

EXPERIMENTAL

Young female mice of the Swiss strain approximately $2\frac{1}{2}$ months of age were used, except for the work on vitamin B supplementation where animals 7–8 months of age were employed. A period of at least two weeks was allowed to elapse before experimental procedures were begun, during which time all animals showing extreme irregularity of estrous cycles were discarded.

The *Lithospermum* used was obtained from the Haskins Laboratories, New York City and was part of that picked in Montana during the 1948 season. The whole plant was fresh-frozen until used except for a brief period when it was removed from refrigeration during shipment to this laboratory, and during which period it underwent "fermentation" of unknown type with considerable heat production. Assay showed this material to be active when fed to mice at a level of 7% dry weight, mixed with Purina Laboratory Chow and baked into pellets under infra-red light. This level of administration was used for the work on the pituitary potency of normal and castrated animals. Later the potency of the *Lithospermum* declined and for the vitamin B supplementation work it was necessary to use a level as high as 30–40% dry weight. Animals received diet and water ad libitum.

The vitamin B supplement used[3] consisted of the following mixture: thiamine HCl, 0.8 mg.; niacin, 4.0 mg.; riboflavin, 1.6 mg.; pyridoxin, 0.8 mg.; and calcium pantothenate, 4.4 mg. A total of .225 mg. of this mixture was given to each animal daily per os in aqueous solution by means of a calibrated dropper.

Castrations were performed bilaterally in one step. Vaginal smears

[3] The author is indebted to Dr. H. G. Day of the Biochemistry Department, Indiana University for supplying these vitamins.

were made daily by the lavage method. Animals were sacrificed by decapitation under light Nembutal anaesthesia and the organs removed, weighed, and fixed immediately. Pituitaries were weighed moist and then acetone-dried and assayed on immature 19 day old female mice according to the method of Riley and Fraps (1942), sacrificing after a period of four days and saving uteri, vaginae, and thyroids for histological examination. Thyroids were sectioned at 5 micra, stained with hematoxylin-eosin-azure, and the height of the follicular cells measured under oil immersion with the aid of an ocular microm-

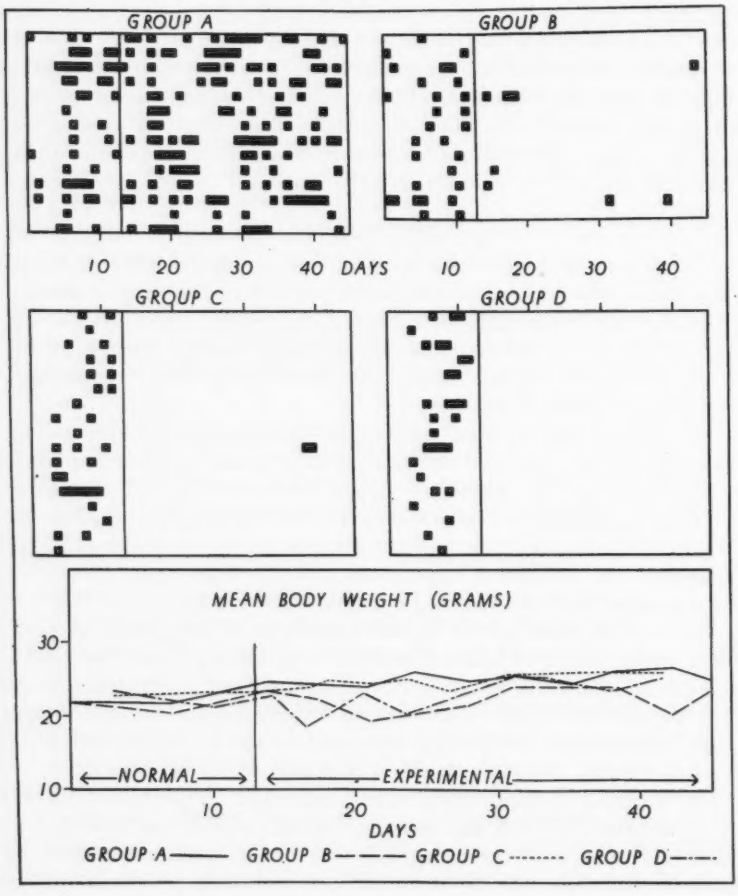

FIG. 1. Vaginal smear and body weight data from intact and castrated animals on normal and *Lithospermum* diet. Group A—intact, normal chow; Group B—intact, *Lithospermum* diet; Group C—castrated, normal chow; Group D—castrated, *Lithospermum* diet. Blackened squares indicate vaginal cornification. The period before the heavy vertical line indicates preliminary control period.

eter. Five animals were chosen at random from each of the experimental groups for this analysis, forty cells being measured in each animal.

RESULTS

Fig. 1 shows the result of *Lithospermum* administration upon the estrous cycles of normal and castrated female mice. It will be noted that this level of *Lithospermum* effectively inhibits the normal cycle, except for the recurrence of estrus in two of the animals toward the end of the experimental period (Group B) which may be due to the phenomenon of refractoriness mentioned in an earlier paper (Drasher and Zahl, 1946). The mean body weights of these animals are seen to fluctuate in an insignificant manner throughout the experiment. At the end of an experimental period of 31 days the animals were sacrificed. Organ weights of these animals are shown in Table 1 in terms of milligrams per 100 grams of body weight to correct for weight discrepancy. Weights of the ovaries and uteri of the intact animals are decreased significantly by *Lithospermum*. Adrenals, thyroids, and thymus show no change. Among the castrated animals (Groups C and D) the only organ exhibiting a significant change is the thymus which has increased in weight as would be expected following removal of estrogenic inhibition. However, it will be noted that the thymic weight of the castrated animals on *Lithospermum* diet is significantly lower than the thymic weight of the same type of animal on a normal diet. This difference is significant at only the 5% level and may be due to some other factor, such as slight inanition. Pituitary weights are given before and after acetone drying and, since the pituitaries were pooled for weighing, the figure given is a mean, uncorrected for body weight. This pooling does not lend itself well to reliable statistical analysis and for that reason no such analysis has been made. The decrease in the pituitary weights of intact animals receiving *Lithospermum* is apparent and is similar to that obtained by Cranston and Robinson (1949). However, it is of interest to note that the pituitary weights of both groups of castrated animals, while exhibiting the typical post-castration rise in weight, do so uniformly and without giving evidence of the inhibitory effect of *Lithospermum* which would be expected if this material were inhibiting gonadotrophin production.

The results of assay for gonadotrophin content of these pituitaries are shown in Table 2. The animals received the total dosage of acetone-dried powder of 0.4 mg. Later it was considered necessary to correct the weights obtained in terms of a dosage of equivalent number of pituitaries, so that assay animals receiving pituitary powder from castrated donors received the equivalent of one gland, and those receiving pituitary powder from intact donors received the equivalent of two glands. On this basis, it is obvious from the table that the results obtained by assay bear out the data on pituitary weights. Uteri from animals receiving pituitaries from normal intact animals are sig-

TABLE 1. ORGAN WEIGHTS FROM INTACT AND CASTRATED ANIMALS ON NORMAL AND *Lithospermum* DIETS

Group	Number of Animals	Ovaries	Uteri Vagina	Adrenals	Thyroids	Thymus	Pituitary	
							Wet	Dry
Control	14	46.07 (3.51)	261.50 (31.02)	53.28 (2.43)	32.00 (2.78)	221.41 (16.86)	2.8	.34
7% LS	12	** 31.00 (2.49)	** 146.91 (13.79)	55.58 (1.02)	26.41 (1.94)	194.55 (15.11)	1.9	.20
Castrated Controls	17	—	82.17 (5.49)	51.29 (2.39)	29.00 (1.80)	** 309.58 (14.56)	3.9	.40
Castrated 7% LS	17	—	95.70 (4.83)	61.35 (2.27)	30.70 (1.78)	* 269.06 (10.66)	3.5	.38

Weights in this table are in mg. per 100 g. body weight, except pituitary weights which are unadjusted. ** indicates a significant difference beyond the 1% level, * a difference at the 5% level as calculated by the "z-test" for significance.[4] Figures in parentheses following the weights indicate the standard error of the mean.

[4] R. A. Fisher's transformation to test significance of differences of standard deviations when N's are small. See Croxton, F. E. and Cowden, D. J., *Applied General Statistics* pp. 344–359, Prentice Hall, New York, 1945.

nificantly heavier than those from assay animals receiving pituitaries from intact *Lithospermum*-fed animals. It is again apparent that *Lithospermum* administration produces no effect on the elevated gonadotrophin potency of the pituitary of the castrated animal, since the uteri from assay animals injected with the pituitaries from castrated animals on either experimental or normal diets show an increase in weight of similar magnitude. Because of this superficial paradox, the ovaries and uteri of both donor and assay animals were studied carefully.

Histological examination of the ovaries of the intact donor animals which had received *Lithospermum* showed smaller ovaries than those

TABLE 2. ORGAN WEIGHTS FROM ASSAY ANIMALS RECEIVING PITUITARIES FROM INTACT AND CASTRATED MICE ON NORMAL AND *Lithospermum* DIETS

Group	Number of animals	Ovaries	Uterus vagina	Thyroids
Blank	6	6.23 (0.54)	17.81 (1.58)	5.91 (0.42)
Control	6	**8.32 (0.37)	**23.30 (1.75)	5.68 (0.35)
7% LS	6	5.78 (0.54)	20.06 (1.00)	5.00 (0.54)
Blank	7	**5.81 (0.50)	**15.81 (0.69)	4.93 (0.12)
Castrated Control	8	7.53 (0.57)	63.02 (3.61)	4.06 (0.33)
Castrated 7% LS	7	8.25 (1.03)	60.02 (2.27)	4.21 (0.57)

Weights in this table are in mgs., unadjusted for body weight. ** Indicates statistical significance beyond the 1% level by the "z-test." Figures in parentheses following the weights indicate the standard error of the mean.

from animals on a normal diet. However, the ovaries from *Lithospermum*-fed animals contained what appeared to be completely functional follicles with no sign of the atresia reported earlier (Drasher and Zahl, 1946). The only difference between the ovaries of these two groups was the complete absence of luteinization in the ovaries of the *Lithospermum*-fed animals. Examination of the uteri gave results which correlated well with the picture obtained in the ovaries. Uteri were graded with respect to degree of glandular development on a scale of one to four, grades 3 and 4 showing definite progestational development. The uteri of the *Lithospermum*-fed animals, of course, had some uteri classified as either grade 1 or 2 (actually only two of the animals) but in these cases examination of both the estrous smear and ovarian histology indicated the estrogenic phase of the estrous cycle.

The ovaries of the assay animals which received pituitary powder from both *Lithospermum*-fed and normal diet castrated animals showed follicular development beyond that found in the assay blanks, but had not undergone luteinization. Likewise, the uteri of these animals did not give evidence of extreme glandular development, although thickness of the endometrium was increased. The vaginae of the assay animals injected with pituitary powder from intact animals, normal diet or *Lithospermum*-fed, showed early stages of stimulation

with no cornification of the epithelium, while vaginae from animals injected with pituitary powder from both groups of castrated animals exhibited complete epithelial cornification.

The results of the histometric analysis of the donor thyroids are presented in Table 3. The results of this analysis indicate that thyroid

TABLE 3. HISTOMETRIC ANALYSIS OF THE FOLLICULAR EPITHELIUM OF THYROIDS FROM INTACT AND CASTRATED ANIMALS RECEIVING NORMAL AND *Lithospermum* DIETS

$$F = \frac{66.65}{45.47} = 1.465 \text{ (not significant)}$$

	Degrees of freedom	Sum of squares	Mean square
Variance between cells	780	3547.0	45.47
Variance between animals	16	789.47	49.34
Variance between treatments	3	199.97	66.65

The method employed in this analysis was that of Snedecor (1948) for the analysis of variance with multiple classification.

implication can be conclusively eliminated from consideration since no significant difference in the heights of the follicular cells could be detected.

The results of vitamin B supplementation are shown in Fig. 2. Group C, receiving *Lithospermum* alone, shows a definite inhibition of estrous cycles when compared with the two control groups, A and B, receiving normal chow and normal chow plus vitamin B supplement respectively. This inhibition is not as complete as that obtained in the earlier series (Fig. 1) and is probably due to the decrease in *Lithospermum* activity mentioned earlier. That excess (adequate B complex was supplied in the normal chow of which the experimental diet was largely composed) vitamin B does not decrease the effectiveness of *Lithospermum* administration, is evident.

DISCUSSION

Results presented above indicate that some explanation of *Lithospermum* activity other than a complete suppression of gonadotrophin production must be found. Indeed, the fact that treated animals return to normal estrous cycles with ease when removed from such a diet even after having been fed *Lithospermum* for relatively long periods of time (Cranston and Robinson, 1949) would cast doubt upon such a hypothesis. The presence of normal follicles with well-developed antra in *Lithospermum*-fed animals suggests that the pituitary is not completely functionless. That *Lithospermum* does not inhibit the post-castration rise in gonadotrophins is further evidence of this. The presence of a complete diestrus in treated animals argues for the lack of estrogenic stimulation, and on the basis of generally accepted ideas of pituitary control of ovarian secretion, viz. that FSH induces secretion of estrogen, LH induces secretion of progesterone, this would point to a suppression of the production of both FSH and LH.

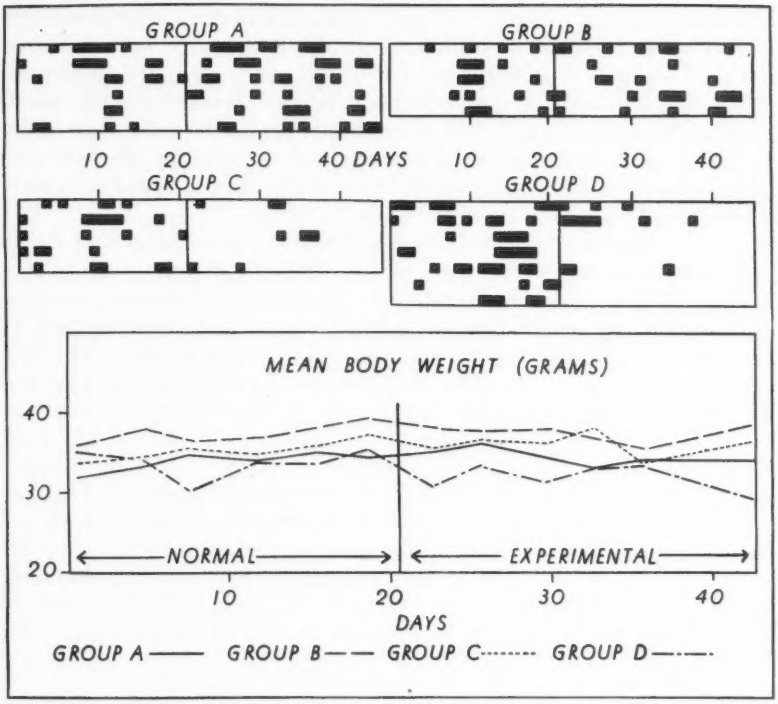

FIG. 2. Vaginal smear and body weight data from intact animals showing the effect of vitamin B supplementation on *Lithospermum* administration. Group A—normal chow; Group B—normal chow, vitamin B supplement; Group C—*Lithospermum* diet; Group D—*Lithospermum* diet, vitamin B supplement. Blackened squares indicate vaginal cornification. The period before the heavy vertical line indicates preliminary control period.

However, it is widely recognized that at present our knowledge of precisely when FSH enters the picture of follicular development and to what degree synergistic action with LH is necessary for the production of significant amounts of estrogen, is incomplete. This is largely due to the difficulty which the proteinaceous nature of the pituitary hormones presents to adequate and accurate fractionation into FSH and LH components. Greep, van Dyke, and Chow (1942) using highly purified thylakentrin (FSH) have shown that in hypophysectomized rats FSH stimulation alone deos not produce secretion of estrogen by the ovary. However, the presence of ovarian follicles containing definite antra is evidence of FSH stimulation. This is precisely the picture encountered in the animals reported upon above.

Hellbaum and Greep (1940) have studied the changes in the pituitaries of rats following castration in a quantitative manner and found that although an increase in gonadotrophin potency occurs shortly thereafter, this increase is largely due to an accumulation of FSH, LH

not being present in detectable amounts until 15 days after castration at the earliest. Since the animals reported upon above were castrated for only 31 days, it is likely that LH would not be expected to be present in large amounts and any decrease in the LH content of the pituitaries of the castrated, *Lithospermum*-fed animals would not be easily demonstrable. Histological examination of the ovaries of assay animals receiving such pituitaries also gives evidence for a predominantly FSH stimulation, no luteinization being found.

A consideration of the above has led to the hypothesis that *Lithospermum* exerts its effect by a specific inhibitory action upon the production of the luteinizing hormone of the anterior pituitary, allowing the secretion of the follicle-stimulating hormone to remain close to normal. This would account for the maintenance of the ovary in a pre-ovulatory state so that ovulation and normal estrous cycles could be resumed rapidly after the cessation of *Lithospermum* treatment, as is the case. Likewise, if it is assumed that small amounts of LH must be present to act synergistically with FSH for the secretion of estrogens by the ovary in amounts sufficient to produce vaginal cornification, the persistent state of diestrus in *Lithospermum*-fed animals can be explained without necessitating the postulation of a reduced FSH output by the pituitary. Whether this hypothesis is correct can only be shown by further experimentation on the quantitative changes in the gonadotrophin potency of animals castrated for periods longer than one month. Such work is now in progress.

SUMMARY

Lithospermum ruderale was fed to normal and castrated animals. While it was found to decrease the gonadotrophin titer of the pituitaries of intact animals, no such diminution was observed in the characteristically elevated gonadotrophin potency of the pituitaries of the castrated animals.

The possibility of indirect action via the thyroids or a borderline deficiency of the vitamin B complex has been negated.

The hypothesis of *Lithospermum* action by means of a specific inhibition of the production of the luteinizing hormone of the anterior pituitary has been suggested.

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THE EFFECT OF GROWTH HORMONE PREPARATIONS ON ALKALINE PHOSPHATASE OF THE TIBIA

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EVIDENCE that anterior pituitary growth hormone stimulates growth of bone is quite extensive. For example, Young (1945) obtained a 6% increase in tibial length by injecting a growth preparation for 60 days into plateaued female rats fed *ad lib.* Becks, *et al.* (1946) found that administration of growth hormone to hypophysectomized rats stimulated bone formation, even after a postoperative interval of one year. The width of the proximal epiphyseal cartilage of the tibia of hypophysectomized rats is used to assay the growth hormone (Evans, *et al.* 1943). Related evidence is the fall in urinary phosphate in dogs (Gaebler and Price, 1937), increase in serum inorganic phosphate in rats (Li, Geschwind, and Evans, 1949), and increase in plasma alkaline phosphatase in rats (Li, Kalman, and Evans, 1947), after growth hormone treatment. Since Robison (1932) established a relationship between bone alkaline phosphatase and bone calcification, and Gomori (1943) confirmed this relationship histochemically, it appeared of interest to investigate the effect of growth hormone upon bone alkaline phosphatase in rats.

EXPERIMENTAL

Normal and hypophysectomized[1] Sprague-Dawley rats of each sex and several age levels were used. In the preliminary experiment, and that reported in Table 1, the rats were fed Rockland rat diet. Work presented in Tables 2 and 3 was carried out with the diet of Bennett, *et al.* (1948). During the final experiment (Table 4), the diet consisted of: casein 24%, white flour 55.5%, whole milk powder 10%, $CaCO_3$ 1.5%, NaCl 1%, and corn oil 8%. Vitamins were added according to Gordan, *et al.* (1948), except that vitamins A and D were given as 2 ml. of haliver oil (60,000 U.S.P. vitamin A units and about 1000 U.S.P. vitamin D units per gm.) per kg. of diet. Both *ad lib.* and paired-feeding techniques were employed, as indicated in the text.

Growth hormone preparations[2] were obtained as lyophilized

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[1] The hypophysectomized rats were obtained from the Hormone Assay Laboratory, Inc., Chicago, Illinois.

[2] The authors are indebted to Dr. D. A. McGinty and Mr. L. W. Donaldson of Parke, Davis & Company for three lots of growth preparation, which had been assayed on plateaued rats. These preparations are about one half as active as the most potent ones obtained by the above investigators to date.

TABLE 1. THE EFFECT OF LARGE DOSES OF GROWTH HORMONE ON TIBIA WEIGHT AND ALKALINE PHOSPHATASE OF MATURE MALE RATS

	No. of rats	Average wet wt. of tibia in gm.	Average alkaline phosphatase concentration of tibia in U./gm.
Injected*	3	0.806	17.6
Control	5	0.804	21.2

* 10 mg. growth preparation (1740 R.U./gm., proven quite potent in promoting nitrogen storage in dogs), was injected daily per rat for 14 days.

powders. For use, this material was suspended in 0.8% saline and injected intraperitoneally. Solutions were prepared fresh every third day and kept frozen at −18°C. when not in use.

All rats were sacrificed at the end of an experiment by a blow on the head, followed by decapitation and exsanguination. The hind legs were amputated and stored at −18°C., the muscle being left in place to prevent dehydration. For analysis, the tibias were dissected out, weighed immediately (wet weight), and reduced to small fragments, either by grinding in a mortar with 1 gm. of quartz sand and 1 to 2 ml. of water, or by freezing with liquid air in a steel mortar and pulverizing with a sharp blow on the pestle. This material was transferred to a graduated tube with the aid of distilled water and diluted, the final volume in ml. being 10 times the number of tibias pooled. Four drops of chloroform were added for each 10 ml. of final solution. The tube was tightly stoppered, and the contents allowed to autolyze at room temperature for 1 to 2 weeks with daily inversion of the

TABLE 2. THE EFFECT OF GROWTH HORMONE ON TIBIA WEIGHT AND ALKALINE PHOSPHATASE OF IMMATURE HYPOPHYSECTOMIZED* FEMALE RATS

	No. of rats	Average‖ initial body wt. in gm.	Average wt. gain in gm.	Average tibia wt. in gm.	Average tibia alkaline phosphatase in U./gm.
I. Hypophysectomized‡					
A. Treated	6	154	52.8	0.402	31.6
B. Control	5	146	− 1.2	0.337	18.7
II. Normal					
A. Treated	5	175	24.6	0.419	27.3
B. Control	5	165	− 0.6	0.405	23.9
P (IA, IB)†			<0.01	<0.01	
(IIA, IIB)			<0.01	>0.3	

* Two months old at operation. Treatment with 1 mg. growth hormone daily per rat (1384 R.U./gm.) for 17 days started 2 weeks after the operation.

‡ Group IA and IB were fed *ad lib.* Group IIA was pair-fed against Group IA, and Group IIB was pair-fed against Group IB.

† Fisher's (1948) table of t. The symbols in parenthesis indicate the groups compared.

‖ Weight at start of injection period.

TABLE 3. THE EFFECT OF GROWTH HORMONE ON TIBIA WEIGHT AND ALKALINE PHOSPHATASE OF IMMATURE, PAIR-FED, HYPOPHYSECTOMIZED* FEMALE RATS

	No. of rats	Average initial body wt.‡	Average wt. gain in gm.	Average tibia wet wt. in gm.	Average tibia alkaline phosphatase in U./gm.
I. Hypophysectomized					
A. Treated	5	176	50.0	0.437	32.4
B. Control	7	173	4.0	0.389	15.5
II. Normal					
A. Treated	5	189	25.0	0.459	24.4
B. Control	6	191	5.0	0.464	25.6
P (IA, IB)†				<0.01	
(IIA, IIB)				>0.7	

* Treatment same as in Table 2, except that a 24 day injection period was employed and all groups were pair-fed against group IB.
† Same significance as in Table 2.
‡ Weight at start of injection period.

tubes. Samples were taken several times during this period to obtain results at maximum extraction. After suitable dilution of this stock enzyme solution, phosphatase activity was determined by the King-Armstrong procedure as modified by Kochakian (1945), the pH of the substrate being 9.1. A Coleman Junior photoelectric colorimeter was employed to read the optical densities at a wave-length of 660 mμ. Activity units are expressed in terms of mg. phenol liberated/hr.

RESULTS

In a preliminary experiment on plateaued female rats, 3 injected animals were restricted to the food intake of 3 untreated controls. Each of the former received 6 mg. of growth preparation (1740 R. U./gm.) daily for 7days. There was no significant increase in the weight or alkaline phosphatase of the tibias. In the next experiment (Table 1) 8 male rats, 8 to 10 months old and fed *ad lib.*, were used. Differences between treated and untreated animals are not informa-

TABLE 4. THE EFFECT OF SMALL DOSES OF GROWTH HORMONE ON TIBIA WEIGHT AND PHOSPHATASE OF IMMATURE, HYPOPHYSECTOMIZED,* PAIR-FED FEMALE RATS

	No. of rats	Average tibia wet weight in gm.	Average tibia alkaline phosphatase in U./gm.
Treated†	10	0.231	31.7
Control	10	0.190	23.8
P		<0.01	

* One month old at hypophysectomy. Plateaued 10 days before starting growth hormone therapy.
† 0.05 mg. growth hormone (1361 R.U./gm.)/rat/day for 6 days, then 0.1 mg./rat /day for 5 days.

tive, as regards tibial weight and alkaline phosphatase. The increase in body weight is not decisive. This is in agreement with the well-known refractoriness of the male rat to growth hormone treatment (Evans and Simpson, 1931). In our limited series, plateaued animals of either sex did not respond quickly with respect to tibial weight. It was, therefore, decided to carry out the rest of the investigation on the much more sensitive, immature hypophysectomized animal.

Results of an experiment, using normal and hypophysectomized female rats, two months old, are recorded in Table 2. The increase in wet weight and alkaline phosphatase of the tibia in the hypophysectomized animals treated with growth hormone is quite striking. Alkaline phosphatase almost doubled, while the tibial weight increase was equal to 27 days of normal growth in the controls. Normals which were pair-fed against the two groups of hypophysectomized animals show much smaller differences, which, as will be shown later, might well be due to the difference in food intake. Group IIA received on an average 2 grams more food per day during the injection period than the untreated normal group IIB. It is to be noted that the hypophysectomized untreated animal has a bone phosphatase level lower than that of the mature males employed in the experiment reported in Table 1. This might be taken to indicate that the bone phosphatase level in these animals is below normal maintenance levels as regards bone formation. The somewhat higher value observed in the normal controls would be indicative of normal bone development in the young and growing animal.

The next experiment was designed to eliminate the variations in food intake. The four groups were arranged as in the previous experiment. Hypophysectomized controls were fed *ad lib.*, while the remaining three groups were pair-fed against this group. The results (Table 3) show the same general trend as in the previous experiment, in which the treated hypophysectomized animals were fed *ad lib.* Untreated hypophysectomized animals have the same low level of alkaline phosphatase activity. Treatment of the hypophysectomized animals doubled the concentration of phosphatase, increasing it above the normal control level. Restricted food intake had no tendency to depress the response. In keeping with the phosphatase response, the low tibia weight due to hypophysectomy was restored to a great extent during the period of growth hormone therapy. In this experiment (Table 3), there is no great difference in phosphatase concentration between the two groups of normal animals. With the food intake equal, the difference previously obtained (Table 2) was reduced and inverted.

As the dosage of growth hormone used in the above experiments was intended to give maximal responses, it might be argued that a "physiological" level of hormone therapy would show no such change. Consequently, an experiment was carried out in which each of 10 hy-

pophysectomized rats received a total of 0.8 mg. of growth hormone preparation in divided doses over a period of 11 days. From the results of this experiment (Table 4), it is obvious that the effect of growth hormone therapy on the wet weight and alkaline phosphatase concentration of the tibia occurs at the low dosage employed, which produced submaximal weight responses averaging 1.3 gm. per rat per day. This is in accord with the extreme sensitivity obtained by Kibrick, *et al.* (1941) in the assay of the growth hormone by the epiphyseal disc method.

DISCUSSION

Studies on the effects of growth hormone preparations on eight enzyme systems are in various stages of completion in this laboratory, and indicate that results of the following types may be expected: first, changes in concentration of enzymes or substrates which are readily correlated with known effects of the hormone; second, changes in which the enzyme may be regarded as an individual protein participating in a general increase in protein synthesis; third, changes having no apparent significance at the moment. Results of the present experiments are clearly in the first category. If one accepts the view of Robison (1932) regarding the role of alkaline phosphatase in bone formation, the large increase of this enzyme in the tibia which we observed in hypophysectomized rats treated with growth hormone would be anticipated, since the action of the hormone on skeletal growth is well known. The expectations of Li and Evans (1947) that the phosphatase concentration in tissues of normal and hypophysectomized rats would be increased by growth hormone are borne out in the case of bone phosphatase in hypophysectomized, but not in normal animals. Results for acid and alkaline phosphatase in liver and kidney, which will be published later, indicate that the changes in concentration of these enzymes in different tissues are not necessarily parallel.

SUMMARY

In immature hypophysectomized female rats, the alkaline phosphatase concentration in the tibia is lower than in normal mature male rats; it is greatly increased by maximal and submaximal doses of growth hormone, and may even be elevated above the level for normal growing animals of the same age and sex. The rise in tibia phosphatase concentration appears to be related to the increase in tibia weight resulting from treatment. Mature and immature normal animals do not respond significantly in these respects, under the conditions employed.

ACKNOWLEDGMENTS

We wish to thank Dr. Paul D. Bartlett for tissues from animals used in a study of the relationship of growth hormone to the gluta-

mine-glutaminase system (Bartlett, 1949) which were made available for the experiment shown in Table 4 of this paper.

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ADRENAL CHANGES PRODUCED IN RATS BY INFECTION WITH *B.* TULARENSE AND *B.* COLI[1]

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IT IS WELL known that the adrenal glands respond to many infectious diseases by a depletion of their cholesterol content (Sayers, Sayers, Fry, White, and Long, 1944) and that this indicates, along with a falling adrenal ascorbic acid content, a state of increased adrenal activity and, if advanced enough, of adrenal exhaustion (Long, 1947). It is also known that infection with tularemia causes pathological changes in the adrenals (Lillie and Francis, 1936) and that these changes are compatible with the pathological picture of adrenal exhaustion (Pinchot).

Quantitative studies of the functional reserve of the adrenal cortex in tularemia, the effect of treatment with cortical hormone, and studies of the respiratory metabolism of animals infected with this disease have not been reported. It was felt that these studies might throw some light on the mechanisms by which this parasite produces its characteristic disease and symptomotology in the host. *B.* coli was used to compare the effects of tularemia with those produced by a less pathogenic gram negative micro-organism.

TECHNIQUE

Male rats of the Sprague-Dawley strain (weighing from 210–290 g.) were used throughout. They were fed a diet of Purina Dog or Laboratory Chow Checkers, supplied with water *ad libitum* and housed in a constant temperature room at $80 \pm 2°$ F.

In the adrenal studies the control values were obtained by removing the glands from normal animals deeply anesthetized with subcutaneously injected "Nembutal." The glands of infected animals were removed in a similar manner or very shortly after death. After removing fat and connective tissue, the glands were weighed to the nearest 0.1 mg. on a torsion balance. One gland was then used for the determination of the ascorbic acid content by the method of Roe and Kuether (1943), modified for tissues, and the other for the cholesterol determination, by the method of Schonheimer and Sperry,

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[1] This work was done under a contract between Yale University and the Special Projects Division of the Chemical Warfare Service.

[2] National Research Council fellow in the Medical Sciences.

as reported by Sperry (1938). Results are reported as the average of duplicate determinations.

The *B.* tularense organisms used were of a highly virulent strain isolated from a human case of the disease, with virulence maintained by mouse and rat passage.[3] Subcultures were made on dextrose cysteine blood agar 24 hours before the animals were to be inoculated. After 24 hours growth at 37° C., the organisms were suspended in sterile physiological saline and diluted to the approximate concentration desired by turbidometric counts with an Evelyn colorimeter. Plate counts were then done on the suspensions to be injected by the method of Downs, Coriell, Chapman, and Klauber (1946).

The *B.* coli was a highly hemolytic strain isolated from the stool of a patient at the New Haven Hospital. This organism was prepared for injection by growing for 12 hours at 37° C. on beef infusion broth. The bacteria were then centrifuged out of the culture and resuspended in 5 ml. of sterile physiological saline per 100 ml. of culture. This material was injected intravenously.

In the first treatment experiment, "Wilson's Cortical Extract," each ml. of which is equivalent to 75 g. of fresh gland, was given subcutaneously in 2 ml. doses to infected rats 5 times a day. Treatment was started 44 hours after the animals were infected and continued until 96 hours after infection. All animals were fasted 96 hours starting at the time of challenge.

In the second treatment experiment Upjohn's "Lipoadrenal Extract," each ml. of which is equivalent to 2 mg. of compound E(11 dehydro 17 hydroxycorticosterone) and contains 40 rat survival-growth units, was given subcutaneously to infected rats in 0.5 ml. doses every six hours starting at 18 hours after infection and continuing until all animals were dead. Control animals of approximately equal weight (± 6 g.) received equal doses of alcohol extracted sesame oil.

The respiratory gas exchange was determined in a constant temperature room at $80 \pm 2°$ F. by the method of Haldane (1892) on rats fasted for 24 hours. The animals were then allowed food for 24 hours, then infected and food was again withheld until the end of the experiment. Periodic measurements of respiratory exchange were made throughout the infection. Control animals were subjected to the same feeding and fasting regime, so as to be able to distinguish between changes produced by the infection and those produced by prolonged fasting, habituation to the procedure, and so on.

Results are reported as means and standard errors throughout.

RESULTS

Table 1 shows the effects of tularemia on the adrenals of the rat. The control values represent determinations on 14 rats except in the case of the cholesterol values which are from 11 rats only.

It will be seen that there was a marked increase in the size of the adrenals of all infected animals. There was an extreme and consistent fall in the adrenal cholesterol values, while the ascorbic acid contents varied from normal to markedly depleted values. This variation and

[3] This strain, SM24R4, was made available by the Technical Director, Camp Detrick.

TABLE 1. THE EFFECTS OF VARIOUS DOSES OF *B.* TULARENSE ON THE ADRENALS OF THE RAT

Wt. g.	Number of organisms injected	Time of death or sacrifice	Mg. adrenal/100 g. body wt.	Adrenal ascorbic mgs./100 g.	Adrenal cholesterol g./100 g.	Minimum body temperature °F.
14 controls						
273 ± 13.1	None	—	12.6 ± 0.71	436 ± 13.4	4.71 ± 0.23	
Infected Animals						
212	16,500	D 4 days	22.2	348	—	—
236	16,500	D 4 days	35.8	166	—	—
232	16,500	S 8 days	—	427	—	100.8
240	16,500	S 8 days	25.9	242	0.35	100.2
230	16,500	S 8 days	18.5	465	0.74	100.6
238	16,500	S 8 days	—	212	—	100.0
242	150,000	D 70 hours	23.3	414	0.5	—
240	150,000	D 68 hours	21.9	421	0.5	—
*218	185,000	D 74 hours	37.2	143	0.39	—
*264	185,000	D 98 hours	21.7	392	0.52	—
*230	185,000	D 98 hours	23.0	319	0.69	—
*220	1,850,000	D 98 hours	23.0	199	0.47	—
*280	1,850,000	D 74 hours	25.1	294	0.40	—
*270	1,850,000	D 91 hours	23.9	290	0.39	—
†240	1,550,000	D 48 hours	29.1	226	0.55	93.2
†256	1,550,000	D 49 hours	20.8	339	0.57	86.0
†250	1,550,000	D 66 hours	—	—	0.42	91.4
†240	1,550,000	D 66 hours	33.2	160	0.55	93.4
241			25.6 ± 1.5	297 ± 25.2	0.50 ± 0.03	

* Animals fed and kept at room temperature.

† Animals fed and kept at room temperature. These animals received sesame oil and are controls for those treated with lipoadrenal extract.

the much smaller variations in the adrenal to body weight ratio and in the cholesterol content seem to have no relation to differences in the size of the infecting dose of organisms or to survival time.

Table 2 shows the effects of treatment of infected animals with water and lipid extracts of the adrenal cortex. The aqueous extract neither prolonged the survival time of the recipients over the controls nor prevented the depletion of the adrenal cholesterol. The lipoad-

TABLE 2. THE EFFECTS OF TREATMENT OF EXPERIMENTAL TULAREMIA IN RATS WITH CORTICAL HORMONE

Wt. g.	Number of organisms injected	Time of death	Mg. adrenal per 100 g. body wt.	Adrenal ascrobic mgs/100 g.	Adrenal cholesterol g./100 g.	Cortical hormone
247	150,000	94 hours	19.7	333	0.55	aqueous
234	150,000	139.5 hours	16.8	—	0.85	extract
272	150,000	79.5 hours	14.1	289	0.66	extract
*242	1,550,000	96 hours	28.9	176	0.45	lipid
*250	1,550,000	66 hours	25.1	262	0.89	extract
*246	1,550,000	51 hours	21.6	354	0.82	extract
*242	1,550,000	66 hours	27.7	324	0.59	extract
248			22.0 ± 2.1	290 ± 26.4	0.69 ± 0.06	

* Not fasted or kept in constant temperature room.

renal extract similarly failed to prevent depletion of the glands. The survival time of the treated animals was prolonged from the control value of 57.3 ± 5.0 hours to 69.8 ± 9.5 hours in the animals receiving lipid extract, but this is not a statistically significant fact. The rats

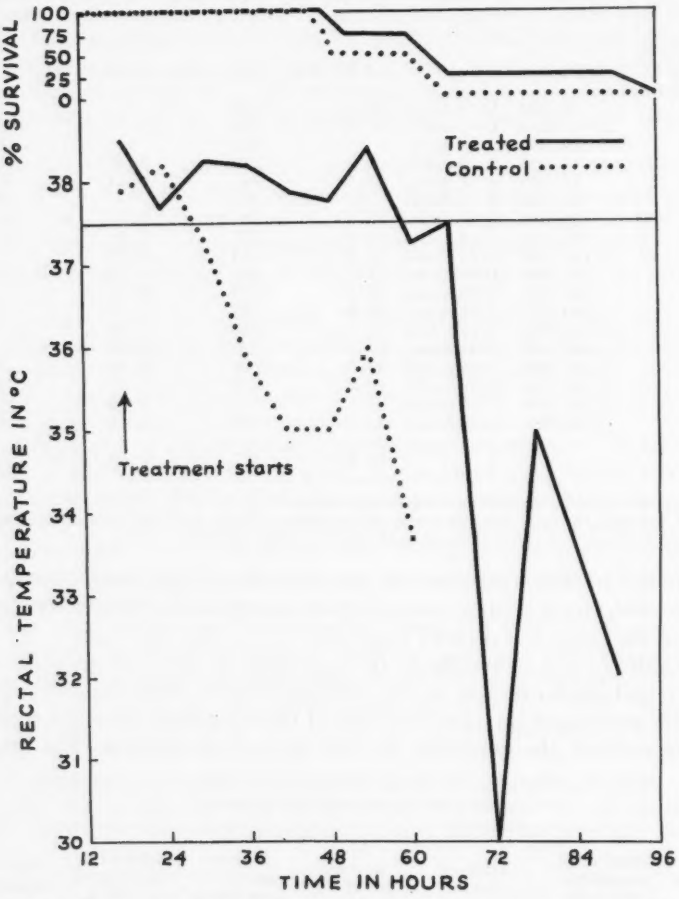

CHART 1. The results of treatment with 0.5 ml. of lipid adrenal extract 4 times per day of rats infected with *B.* tularense.

receiving the lipoadrenal extract were, however, able to maintain their body temperature longer than those which did not receive it, as is shown in chart 1. Rats dying of tularemia have a characteristic and profound fall in body temperature, the onset of which precedes death by from 12 to 24 hours. Moreover, the rats treated with cortical hormone did not seem acutely ill until a few hours before death, while the

controls gave evidence of a severe illness 30 hours after inoculation with *B.* tularense organisms.

There were no consistent differences in oxygen consumption or caloric output between the infected and control groups. The infected group did have consistently higher RQ values than the controls, however. Six animals were used in the control group and ten in the infected. The infected group received from 195 million to 5 billion *B.* tularense organisms. The mean RQ's were as follows: Control determinations; controls .719 ± .0053, animals to be infected .722 ± .0047; 24-hour determination; controls .732 ± .0053, infected .770 ± .0086. P is less than .01; 48-hour determination; controls .735 ± .0054, infected

TABLE 3. THE EFFECTS OF LIVING *B.* COLI INJECTED INTRAVENOUSLY INTO RATS

Wt. g.	Ml. of *B.* coli	Mg. adr./ 100 g.	Time sacrificed or death	Adrenal ascorbic acid mg./100 g.	Adrenal cholesterol g./100 g.	Minimum body temperature °F.
220	0.5 ml.	18.1	S 5 hours	274	1.92	92.0
210	0.5 ml.	16.1	S 4.5 hours	262	2.59	91.0
210	1.0 ml.	15.3	D 5 mins.	329	3.39	—
225	1.0 ml.	17.8	S 5 hours	260	1.84	91.0
216	2.0 ml.	17.6	D 15 mins.	404	3.24	96.0
220	2.0 ml.	14.0	S 2.5 hours	265	1.28	83.5
280	2.0 ml.	12.6	S 4.5 hours	266	2.19	91.0
310	2.0 ml.	12.0	S 4.5 hours	255	2.31	88.5
316	2.0 ml.	11.5	S 3.8 hours	234	1.08	90.0
290	2.0 ml.	12.4	S 4 hours	250	1.86	90.0
259*		14.3 ± 0.94		258 ± 4.3	1.88 ± 0.18	

* The animals dying in 5 + 15 minutes after injection are excluded from the means, as the time was inadequate for their adrenals to show changes in cholesterol and ascrobic acid content.

.783 ± .0159. P is less than .01; 72-hour determination; controls .742 ± .0029, infected .756 (one animal surviving).

The results of inoculation with *B.* coli are shown in Table 3. The animals were sacrificed when in extremis, or when their temperatures began to rise from a low point. This was felt to represent the time of maximal change, since several were allowed to recover after their temperatures began to rise, which they did uneventfully. The inoculations then either produced death in a relatively short time or were self-limiting. It will be seen from the table that the injection of this organism produced a moderate increase in adrenal size, a moderate fall in adrenal cholesterol, and a moderate and consistent fall in adrenal ascorbic acid. There was also a fall in temperature.

DISCUSSION

The adrenal hypertrophy and the profound fall in cholesterol content in rats infected with tularemia probably indicates a marked exhaustion of the glands by this disease. The drop in cholesterol is greater than that produced by a single injection of adrenotropichor-

mone (Sayers, Sayers, Liang, and Long, 1946) and is of the same magnitude as the type III adrenal response described by Sayers, Sayers, Fry, White and Long, (1944) which represents a situation characterized by continuous stimulation and collapse ending in death. The cholesterol values are similar to those reported in humans dying of infectious diseases.

On the other hand, the mean value of the adrenal ascorbic acid content of the infected animals is higher than that observed by the above authors following a single dose of adrenotropic hormone. Some of the levels of the ascorbic acid are perfectly normal in spite of the extremely low cholesterol values and the increased adrenal size. It would therefore seem likely that the adrenal ascorbic acid content may return to normal or near normal values in some cases of prolonged adrenal stimulation. In any case it would appear that this determination alone is not a reliable index of the functional state of the adrenal which has been subjected to a prolonged stress.

It has been shown that pretreatment with cortical hormone prevents the fall in adrenal ascorbic acid and cholesterol content produced by many forms of stress, and it has been suggested that this prevents stimulation of the pituitary by the low blood level of cortical hormones otherwise presumably caused by stress (Sayers, and Sayers 1947). It has also been shown that the rat is able to restore both cholesterol and ascorbic acid levels in the adrenal to normal within 24 hours after a fall produced by adrenotropic hormone (Sayers, Sayers, Liang and Long, 1946).

Vogt (1943) has shown that the adrenals of a ten kilo dog may discharge the daily equivalent of 230 ml. of commercial water extract of the same strength as that used in these experiments. This output is presumably markedly increased by stress, yet in infectious diseases the supply is apparently not adequate to meet the demand as is shown by the exhausted glands found at death. As can be seen from the results, treatment with the available preparations of adrenal cortical hormones, at least in the amounts used in these experiments, not only failed to protect the animals, but also failed to prevent the depletion of the adrenal cholesterol. The answer to the question of whether or not larger doses would protect the adrenals and save the animals must await the availability of more potent preparations of cortical hormone, since the two used here were given in larger doses than could be completely absorbed. The temperature curve and appearance of the animals treated with the lipid preparation suggests that the treatment did help the animals in the early stages of the disease.

In any event these experiments indicate that the requirement for cortical hormone is greatly increased in tularemia. Our animals received 10 ml. of water soluble extract per day (or 400 ml. per kilo of animal for comparison with Vogt's figures) and in the other experiment the equivalent of 4 mg. of compound E or 80 rat survival-

growth units, in addition to the output of their own adrenals, without meeting the demand.

These results suggest the possibility that at least some of the published reports of failures of cortical preparations to benefit various diseases upon which they have been tried may have been the result of inadequate dosage.

The experiments with *B.* coli show the marked differences in the effects produced by a stress of short duration from those produced by a long-term stress, even when both are caused by the injection of bacteria. In the animals receiving the colon organism the levels of adrenal ascorbic acid and cholesterol were quite analogous to those seen following a single injection of pituitary adrenotropic hormone (Sayers, Sayers, Liang, and Long, 1946) or of epinephrine (Long and Fry, 1945). The adrenals showed less evidence of exhaustion as measured by hypertrophy and cholesterol changes than in the animals which received *B.* tularense organisms. This is presumably because of the shorter duration of the adrenal stress in the former experiment, not because of a submaximal stimulus, since adrenotropic hormone does not produce any greater changes in the same time.

The uniform fall in ascorbic acid content (except in the animals dying in 5 and 15 minutes) and the close correlation between ascorbic acid and cholesterol contents show the reliability of the former determination in short experiments such as this. This is in marked contrast with the situation in the animals with tularemia. The fall in body temperature is similar to that seen in animals dying of tularemia.

SUMMARY

Rats infected with *B.* tularense showed an increase in adrenal size and a profound fall in adrenal cholesterol content. The change in adrenal ascorbic acid content was highly variable, indicating the unsuitability of this determination as an indication of the functional state of the adrenal cortex in prolonged infections. Infected rats showed a significant rise in RQ as compared with normals.

Dosages of adrenal cortical hormone preparations many times larger than those required for maintenance of adrenalectomized animals failed to prevent the adrenal cholesterol depletion or significantly prolong the survival time of infected animals, although the treated animals were better able to maintain body temperature and seemed more active than the controls.

Rats receiving large doses of living *B.* coli intravenously had an adrenal cortical response similar to that produced by a single injection of pituitary adrenotrophic hormone as measured by adrenal size, ascorbic acid and cholesterol content. The ascorbic acid levels correlated with the other measurements of adrenal cortical function in this short-term experiment.

The differences in the effects of the two organisms on the adrenals

are believed to be primarily differences in the duration of the adrenal stimulation they produced.

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THE EFFECT OF ADRENAL CORTEX EXTRACT UPON URINARY NON-PROTEIN NITROGEN AND CHANGES IN WEIGHT IN YOUNG ADRENALECTOMIZED RATS

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THE ADRENALECTOMIZED rat is unable to grow at a normal rate (Hartman and Thorn, 1930). In the present study we have tested the suggestion (Ingle, 1944) that the slowing of growth in the adrenally insufficient animal is due entirely to secondary changes, principally the reduction in intake of food. Adrenalectomized rats were given a constant caloric intake by stomach tube and received saline to drink. The administration of adrenal cortex extract was followed by a decrease in urinary non-protein nitrogen and an increase in rate of gain in weight as compared to adrenalectomized rats which did not receive the hormone.

METHODS

Male rats of the Sprague-Dawley strain were maintained on Archer Dog Pellets until they reached a weight of approximately 300 grams. At this time they were placed in metabolism cages and adapted to the force-feeding of a medium carbohydrate diet by stomach tube each morning (8:30 to 9:15 A.M.) and afternoon (4:15 to 5:00 P.M.). The technique of force-feeding and the diet (Table 1) used were modifications of those described by Reinecke, Ball and Samuels (1939). The animals were brought to a full feeding af 26 cc. per rat per day on the 5th day. After the rats had been force-fed for 2 weeks the adrenal glands were removed by the procedure of Ingle and Griffith (1942). Asepsis was successfully maintained in these operations. All of the rats were given a solution of 1 per cent sodium chloride to drink during all phases of the experiments. The experiments were done in air-conditioned rooms in which the temperature was maintained at 74 to 78 degrees F. and the humidity at 30 to 35 per cent of saturation. Beef adrenal extract (ACE) was used which was free from alcohol and represented 40 grams of gland per cc. It was administered subcutaneously in divided injections twice daily.

Beginning with the third week after adrenalectomy, 24-hour samples of urine were collected each morning prior to feeding. The urines were preserved with thymol and added citric acid (1 g. per sample) to insure the acidity of the urines for nitrogen analysis. The determination of urinary non-protein nitrogen was by the micro-Kjeldahl procedure. In Experiment 1, the determinations were made on each 24-hour sample of urine. In Experiment 2, the urines were pooled into 72-hour samples for each rat.

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EXPERIMENTS AND RESULTS

In Experiment 1 (Figure 1), 22 adrenalectomized rats were observed during a control period of 14 days. Eleven of these rats were then given 1 cc. of ACE for 7 days, 2 cc. for 7 days, 3 cc. for 7 days, and 4 cc. for 7 days. The 11 control rats were given equal volumes of 0.9 per cent sodium chloride solution for these periods. There was a

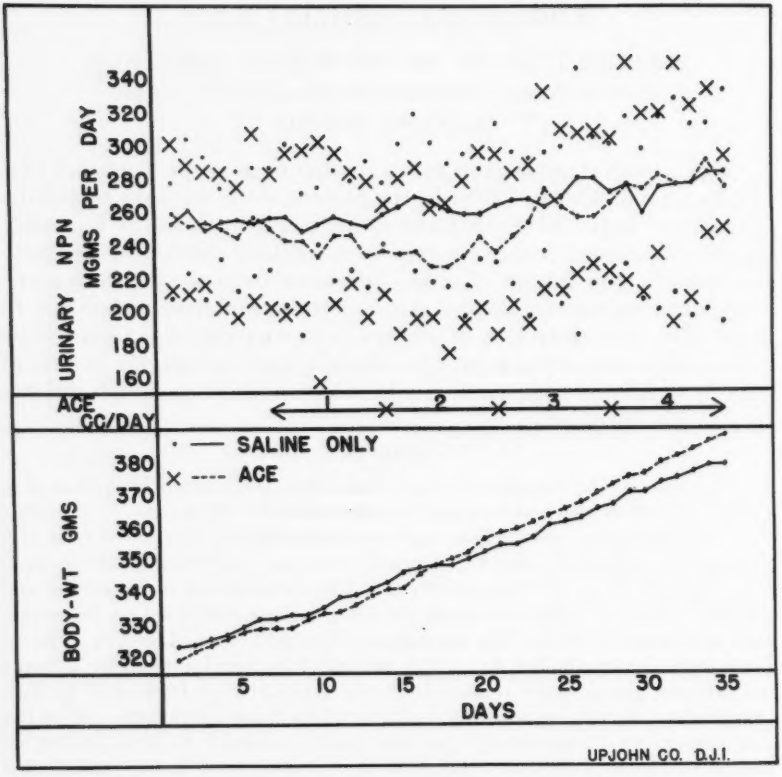

FIG. 1. The effect of adrenal cortex extract upon gain in weight and urinary non-protein nitrogen (averages and range) in adrenalectomized rats. Eleven pairs.

decrease in the excretion of urinary non-protein nitrogen during the administration of 1 and 2 cc. daily of ACE but when the dose was increased to 3 and 4 cc. daily, the level of nitrogen excretion was increased. The group treated with ACE showed greater gains in weight than did the control animals.

In Experiment 2 (Figure 2), 12 adrenalectomized rats were observed during a control period of 14 days. Six of these rats were then treated with 2 cc. of ACE daily for 18 days. The 6 control rats were

given 2 cc. per day of 0.9 per cent sodium chloride solution. There was a prompt decrease in the level of urinary non-protein nitrogen during the administration of ACE and an accompanying increase in the rate of gain in weight.

DISCUSSION

Although the adrenally insufficient rat is capable of growing when maintained on a normal caloric intake and saline to drink, it seems

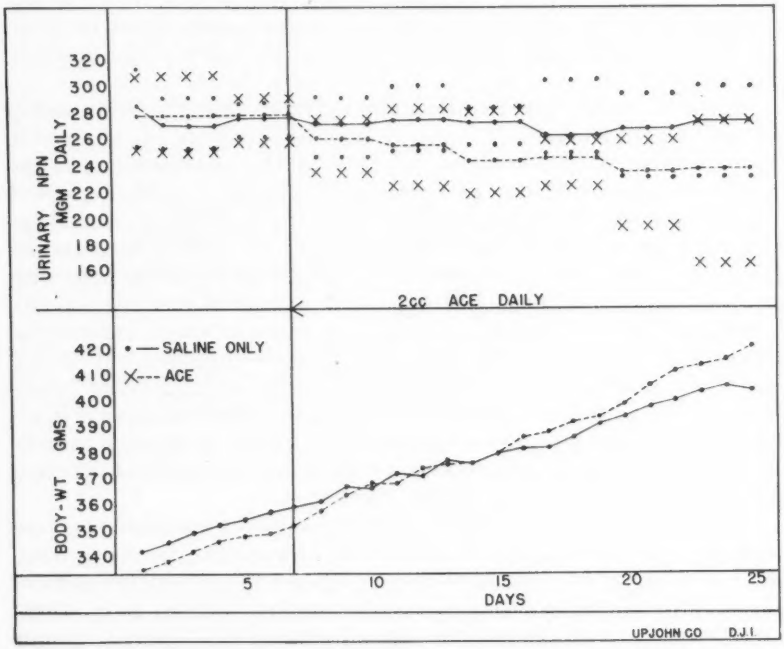

FIG. 2. The effect of adrenal cortex extract upon gain in weight and urinary non-protein nitrogen (averages and range) in adrenalectomized rats. Six pairs.

probable that hormones contained in ACE are essential for the animal to attain an optimal rate of anabolism. A number of other studies (Koelsche and Kendall, 1935; Berman *et al*, 1947; Ingle and Oberle, 1946; Ingle, Ward and Kuizenga, 1947; Ingle and Prestrud 1948) have called attention to experimental conditions in which the adrenal cortical hormones support anabolism. Large amounts of adrenal steroids, especially the 11,17-oxygenated compounds, may cause rapid depletion of some of the body tissues, but it may be correct to characterize these changes as overdosage effects. Several other hormones including thyroxin, androgens and insulin can stimulate catabolism when given in large amounts, although smaller amounts may favor anabolism.

The relationship of the thyroid to growth may be comparable to that of the adrenal cortex. The growth of the thyroidectomized rat is stunted; growth is stimulated by treatment with physiological amounts of thyroxin, but the administration of excessive amounts of this hormone inhibits growth. The changes in nitrogen balance of force-fed thyroidectomized rats parallel the effect of thyroxin upon growth (Rupp and Paschkis, 1948).

Androgens are known to stimulate anabolism. Does ACE contain physiologically important amounts of androgens? The question has not been fully answered but we have never been able to demonstrate an effect of ACE upon the weight of seminal vesicle of the castrate rat.

Ingle (in press) has reviewed the evidence that the relationship of the cortical hormones to growth may be unspecific. It is possible to rationalize at least some of the data by the assumption that the inhibitory effects of the 11-oxygenated adrenal steroids represent overdosage; and by the assumption that the inability of the saline-treated adrenalectomized animal to grow at an optimal rate and its inability to normally accelerate its mobilization of endogenous proteins during stress may each represent a general inability of any metabolic process to reach a maximum response in the absence of the cortical hormones. There may be no complete dropping out of any metabolic process in the adrenally insufficient animal. When conditions of diet and environment are optimal and when the load is light, the rates of many processes approximate those of normal animals at rest but the adrenally insufficient organism is incapable of maximal acceleration of the process under stimulation.

A second hypothesis holds that the hormones of the adrenal cortex such as the 11-oxygenated compounds represent specific growth inhibitors which are normally balanced against the growth hormone of the anterior pituitary. To support this concept (Baker *et al.* 1948) are the many observations that the adrenal cortical steroids inhibit growth and that following adrenalectomy there is increased growth of lymphoid tissue (Reinhardt and Holmes, 1940), hair (Butcher, 1941), and an increase in the growth of bone (Wyman and Tum-Suden, 1945).

Elucidation of the relationship of the adrenal hormones to growth will require further study of the effects of the specific adrenal steroids upon specific tissues.

SUMMARY

Two experiments were carried out upon force-fed adrenalectomized male rats having initial weights of approximately 300 grams. In Experiment 1, 11 adrenalectomized rats were given injections of 1 cc. of adrenal cortex extract for one week, 2 cc. for one week, 3 cc. for one week, and 4 cc. for one week. An equal number of control

animals were given injections of physiological saline. The level of urinary NPN was decreased and the rate of weight gain was accelerated by 1 and 2 cc. of ACE per day but when the dose was increased to 3 and 4 cc. the level of nitrogen excretion was increased.

In Experiment 2, 6 adrenalectomized rats were given 2 cc. of ACE daily for 18 days. There was a decrease in the level of urinary NPN and an increase in the rate of weight gain above that of the 6 control animals.

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EFFECTS OF UNDERFEEDING ON THIOURACIL ACTION IN RATS AND MICE

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THE ABILITY of thiouracil to induce thyroid hypertrophy is believed to be mediated through a direct depression of thyroid hormone synthesis, thereby permitting increased thyrotrophic hormone production by the anterior pituitary and greater stimulation of the thyroid (MacKenzie and MacKenzie, 1943; Astwood *et al.*, 1943). Underfeeding is believed to inhibit thyroid function through a direct depression of thyrotrophic hormone secretion by the anterior pituitary (Mulinos and Pomerantz, 1940, 1941; Stephens, 1940; and others). Thiouracil action therefore might be expected to be less effective in underfed than in normally-fed animals, since the initial thyroid and thyrotrophic hormone secretion rates would be lower than in normally-fed animals.

In a previous report on the effects of starvation on thiouracil action in rats, Gomez Mont, Paschkis and Cantarow (1947) found that the hypertrophy and hyperplasia of the thyroids was less marked and developed later than in normally-fed controls. These workers administered the thiouracil as a 0.05 per cent solution to which the underfed and normally-fed rats had free access. It seems doubtful that by this procedure the underfed rats would have consumed as much of the thiouracil solution as the normally-fed rats, since it is well known that starved animals usually reduce their fluid intake. The senior author (J. M.) has repeatedly observed that in a temperature and humidity-controlled animal room, water intake of rats and mice is more or less proportional to feed intake. It seems possible, therefore, that a reduced thiouracil intake by the underfed rats could account for the results reported by Gomez Mont *et al.* (1947).

In the experiments reported here, the effects of both a constant injected dose and of various levels of orally administered thiouracil were determined in rats and mice on restricted diets. The results indicate that on a 100 gm. body weight basis, the underfed animals which received the same dose of injected thiouracil showed the same increase in thyroid weight as the *ad libitum*-fed controls, while the

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animals which were fed less thiouracil than the controls showed a reduced thyroid response.

METHODS

A total of fifty-eight mature female albino rats of the Michigan State College strain and fifty-nine immature and mature male albino mice of the Rockland strain were used in these experiments. The rats and mice were each divided into groups which received the same dose of thiouracil by injection, or different amounts of feed containing thiouracil.

In the rats, thiouracil administration was started simultaneously with underfeeding, and continued for ten days only. The injected rats received an aqueous suspension of ten mg. of thiouracil daily, given in two divided, subcutaneous injections. The thiouracil-fed rats received the drug in the form of a 0.1 per cent mixture in the feed. The restricted diet rats were given either $\frac{3}{4}$ or $\frac{1}{2}$ of the feed consumed daily by the *ad libitum*-fed controls. On the 11th day, all the rats were sacrificed, and the thyroids were carefully removed and weighed on a Roller-Smith balance.

The mice were similarly put on *ad libitum*, $\frac{3}{4}$ or $\frac{1}{2}$ normal feed regimens, but this was continued for 24 instead of ten days. From the 15th through the 24th day, each mouse was injected subcutaneously, once daily, with an aqueous suspension of 10 mg. of thiouracil, or was given feed containing 0.2 per cent thiouracil. The mice were sacrificed on the 25th day. The thyroid weights of the mice and rats were all subjected to statistical analysis. All animals were maintained in a temperature controlled room at 75°F.

RESULTS

Rats

The data on the rats are summarized in Table 1. It can be seen that the control rats (group 1), fed a thiouracil-free diet *ad libitum*, had thyroids weighing 8.1 ± 0.6 mg. per 100 gm. body weight, while the rats fed the 0.1 per cent thiouracil diet *ad libitum* (group 2) had thyroids weighing 24.4 ± 1.5 mg. per 100 gm. body weight. Groups 3 and 4, which were on $\frac{3}{4}$ and $\frac{1}{2}$ normal feed intakes, and consequently ate only $\frac{3}{4}$ and $\frac{1}{2}$ of the thiouracil consumed by group 2, had thyroids which were significantly smaller in size than the rats of group 2 on a 100 grams body weight basis. Groups 5, 6 and 7, which received the same amount of thiouracil by injection, all showed similar increases in thyroid weight on a 100 gm. body weight basis, regardless of the differences in feed consumption.

Mice

The data on the mice are summarized in Table 2. The thiouracil-fed mature mice will be considered first. The mice of group 1, which were permitted to eat *ad libitum* a diet free of thiouracil, had thyroids weighing 8.1 ± 0.9 mg. per 100 gm. body weight, while the mice of group 2, which were fed *ad libitum* a diet containing 0.2 per cent thiouracil, had thyroids weighing 37.3 ± 1.5 mg. per 100 gm. body weight. The mice of group 3, which received only one-half the amount of feed containing the 0.2 per cent thiouracil consumed by

TABLE 1. EFFECTS OF THIOURACIL ON THYROID OF UNDERFED FEMALE RATS

Group	No. rats per group	Procedure	Orig. body wt. gm.	Final body wt. gm.	Diff. in body wt. %	Thyroid wt. mg.	Thyroid wt. 100 gm. body wt. mg.
		Thiouracil fed					
1	10	Fed *ad lib.* 10 days; no thiouracil in feed	145.0	165.0	+13.8	14.0	8.1 ±0.6*
2	8	Fed *ad lib.* 10 days; 0.1% thiouracil in feed	145.5	143.5	− 1.3	35.0	24.4 ±1.5†
3	8	Fed ¾ *ad lib.* 10 days; 0.1% thiouracil in feed	147.5	128.4	−12.9	26.7	20.8 ±1.4†
4	8	Fed ½ *ad lib.* 10 days; 0.1% thiouracil in feed	145.5	119.8	−17.6	23.0	19.1 ±1.1†
		Thiouracil injected					
5	8	Fed *ad lib.* 10 days; 10 mg. thiouracil injected daily	155.0	162.0	+ 4.5	34.3	21.2 ±1.2
6	8	Fed ¾ *ad lib.* 10 days; 10 mg. thouracil injected daily	158.0	149.2	− 6.2	32.1	21.5 ±1.3
7	8	Fed ½ *ad lib.* 10 days; 10 mg. thiouracil injected daily	157.0	131.0	−15.9	28.8	21.9 ±1.6

* Standard Error of Mean.
† The means from group 3 and 4 are significantly less than the mean for group 2.

TABLE 2. EFFECTS OF THIOURACIL ON THYROIDS OF UNDERFED MALE MICE

Group	No. mice per group	Procedure	Orig. body wt. gm.	Final body wt. gm.	Diff. in body wt. %	Thyroid wt. mg.	Thyroid wt. 100 gm. body wt. mg.
		Thiouracil fed					
1	8	Fed *ad lib.* 24 days; no thiouracil in feed	32.8	35.5	+ 8.2	2.86	8.1 ±0.9*
2	8	Fed *ad lib.* 24 days; 0.2% thiouracil in feed last 10 days	32.0	36.3	+ 13.4	13.62	37.3 ±1.5
3	8	Fed ½ *ad lib.* 24 days; 0.2% thiouracil in feed last 10 days	31.2	25.2	− 19.2	4.16	17.0 ±1.7
		Thiouracil injected					
4	10	Fed *ad lib.* 24 days; no thiouracil injected	9.5	28.3	+197.9	3.11	11.0 ±0.7
5	7	Fed *ad lib.* 24 days; 10 mg. thiouracil injected daily last 10 days	9.1	26.7	+193.4	7.98	30.0 ±5.6
6	10	Fed ¾ *ad lib.* 24 days; 10 mg. thiouracil injected daily last 10 days	10.5	22.2	+111.4	5.79	26.1 ±2.1
7	8	Fed ½ *ad lib.* 24 days; 10 mg. thiouracil injected daily last 10 days	10.3	15.8	+ 53.3	5.53	35.0 ±2.9

* Standard Error of Mean.

group 2, had thyroids which weighed only 17.0 ± 1.7 mg. per 100 gm. body weight, or about one-half the weight of the thyroids of group 2.

The underfed immature mice of groups 6 and 7, which were all injected with the same dosage of thiouracil, showed the same increases in thyroid weight per 100 gm. of body weight as the *ad libitum*-fed controls (group 5). The effects of restricted feed intake on the body weights of these mice was more severe than in the mature mice and rats.

DISCUSSION

The data presented here indicate that underfeeding does not reduce the response of the thyroids to a constant dosage of thiouracil in underfed rats and mice on a body weight basis. The opposite findings of Gomez Mont *et al.* (1947) in underfed rats may well have been due to a lowered thiouracil intake, which was shown here to elicit a smaller response in both rats and mice.

These data do not indicate that underfeeding increases the sensitivity of the thyroids of rats and mice to thyrotrophic hormone, although such a possibility cannot be completely excluded. Stephens (1940) found that the thyroids of starved guinea pigs were more reactive to injected thyrotrophic hormone than normally-fed guinea pigs, but Mulinos and Pomerantz (1941) were unable to corroborate this observation in underfed rats.

The fact that thiouracil can still induce an increase in thyroid size during even severe dietary restriction, as was the case in the mice given one-half of the normal feed intake, indicates that the thyroid gland does not completely cease functioning during starvation. If the thyroids had stopped secreting thyroid hormone altogether, then thiouracil would obviously have had no effect.

The similar increases in thyroid weight per unit of body weight observed in both rats and mice given the same dosage of thiouracil, suggests that thyroid secretion in underfed animals is reduced only in proportion to loss in body weight. Further confirmation of this idea comes from the observation that in intact mature female rats which received either $\frac{3}{4}$, $\frac{1}{2}$ or $\frac{1}{4}$ of the feed consumed by *ad libitum*-fed controls for fourteen days, or no feed for seven days, the weight of the thyroid per unit of body weight remained the same in each case (Meites and Reed, 1949). The thyroidal status during undernutrition, therefore, differs from gonadal and adrenal cortical status, since the gonads may stop functioning entirely while the adrenal cortex usually becomes more active during severe underfeeding.

SUMMARY

The influence of underfeeding on the response of the thyroid gland to either constant or varied amounts of thiouracil was de-

termined in fifty-eight mature female rats and in fifty-nine mature and immature male mice. Thiouracil was injected subcutaneously in the form of an aqueous suspension at the rate of 10 mg. daily or was fed in the ration as a 0.1 or 0.2 per cent mixture. Food intake was restricted by feeding either $\frac{3}{4}$ or $\frac{1}{2}$ of the feed consumed daily by *ad libitum*-fed controls.

It was found that on a 100 gm. body weight basis, the underfed and *ad libitum*-fed animals which received the same amount of thiouracil showed the same increases in thyroid weight, while the animals which received less thiouracil showed reduced thyroid responses. These results suggest that thyroid secretion in underfed rats and mice is reduced only in proportion to loss in body weight.

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INFLUENCE OF THE DIETARY PROTEIN CONCENTRATION UPON THE CORTICOTROPHIC ACTION OF LYOPHILIZED ANTERIOR PITUITARY

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IT HAS BEEN previously reported that the production of nephrosclerosis and hypertension by lyophilized anterior pituitary (LAP) is particularly pronounced on high-protein diets (30%) (Dontigny, Hay, Prado and Selye, 1948; Hay, Prado and Selye, 1948). Further experiments with protein hydrolysates (DeGrandpré, Prado, Dontigny, Leduc and Selye, 1948) and amino-acids (Henriques, Henriques, De Grandpré and Selye, 1948) showed that the latter are responsible for this sensitizing effect of protein. In all these experiments, a marked adrenal enlargement, nephrosclerosis and hypertension were observed in animals treated with LAP and kept on high-protein diets, while slight stimulation of the adrenals, no nephrosclerosis and a normal blood pressure were recorded in similarly treated rats receiving low-protein diets. It was suggested that the hypertension produced by LAP is mediated through the adrenals and kidneys and that the corticotrophic action of hypophyseal extracts is enhanced by dietary protein (Selye, 1946, 1948).

Conversely, the development of hypertension and nephrosclerosis in animals with an "endocrine kidney" (Selye and Stone, 1948) and in those treated with DCA (Prado, Dontigny and Selye, 1947), is not influenced by the protein content of the diet.

These findings suggested that the site of action of dietary protein is between the pituitary and the adrenal cortex; it either increases the efficacy of the corticotrophic hormone (ACTH) or stimulates ACTH production.

It was reported that the ACTH activity of pure preparations is independent of dietary proteins (Ingle, Prestrud, Li and Evans, 1947; Moya, Prado, Rodriguez, Savard and Selye, 1948). Moya *et al.* (1948) showed that under stress ("alarm reaction") the ACTH production, as detected by adrenal ascorbic acid depletion, is increased by diets rich (30%) in protein. It could be that during stress, in addition to ACTH, other corticotrophic pituitary constituents are

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formed and that these are activated by protein. Alternately, protein could augment ACTH-secretion.

In order to gain further information concerning this point, we have studied the effect of dietary protein upon the corticotrophic activity of a total anterior-pituitary preparation (LAP) after hypophysectomy. We used adrenal ascorbic-acid depletion (experiment I) and adrenal enlargement (experiment II) as criteria.

I. INFLUENCE OF DIET ON ASCORBIC ACID DEPLETION ACTIVITY OF LAP

Material and Methods.—Male piebald rats, weighing 120–140 gm. were divided into 2 groups and fed on experimental diets (see table 1) for 15 days. Group I received a 5% casein diet (Diet 1) and group II a 30% casein diet (Diet 3). At the end of this period, all rats were hypophysectomized and 21

TABLE 1. COMPOSITION OF DIETS

Diets	1	2	3
Casein	5	10	30
Starch	88	83	63
Fat	1	1	1
Steenbock's salt mixture	4	4	4
Cod liver oil	1	1	1
Cellu flour	1	1	1
Vitamins*	1 ml.	1 ml.	1 ml.

* Hawk, Oser and Summerson, 1947.

to 27 hours later the corticotrophic activity of LAP was determined by the ascorbic acid depletion method, following exactly the experimental design and schedule recommended by Sayers, Sayers and Woodbury (1948). 2.6 mg. per 100 gm. of body weight of a fresh 1% solution of nembutal was used as anesthetic. For the adrenal ascorbic acid determinations, the method of Roe and Kuether (1943) was employed. The LAP was ground, passed through a no. 18 U. S. standard sieve (1 mm. opening) and carefully homogenized. 100 mg. of LAP were thoroughly extracted with 25 ml. of 0.01N NaOH in 0.9% NaCl and the solution made up to 100 ml. with 0.9% NaCl (stock solution). The solutions used for injection were prepared by appropriate dilution of the stock solution. For each test a new stock solution was prepared. Several preliminary tests were made to obtain the useful dose-range.

The final assay was performed with the doses 3.5, 7 and 14 γ of LAP per 100 gm. of body weight, given by intra-jugular injection.

RESULTS

Chart 1 and table 2 show the dose-response relationship in both groups of animals, as judged by the resulting adrenal ascorbic acid depletion. The data were submitted to variance analysis (table 3) according to Bliss and Marks (1939), the significance of differences of variances being tested through Fisher's z test (Fisher, 1944). The item "samples" in table 3 indicates the variance produced by the use of two different groups of animals, *i.e.*, animals treated with 5%

and 30% casein. It should be observed that the variance produced by "samples" is smaller than the experimental error; therefore an increase of 5% to 30% in dietary protein concentration caused no statistically significant change in the sensitivity of rats to the corticotrophic activity of LAP as observed by the ascorbic acid depletion test. This is further substantiated by computing the ratio of corticotrophic activity of LAP in both groups of animals. The potency

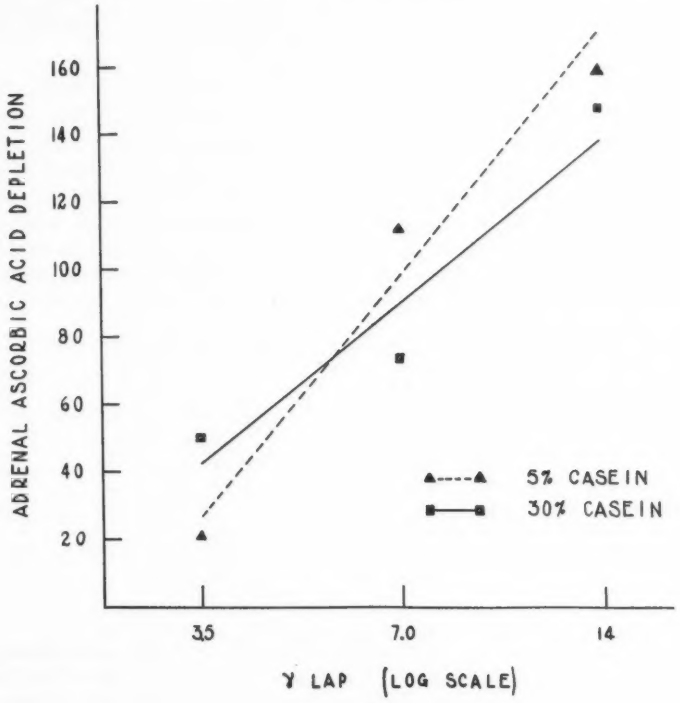

CHART 1

ratio obtained was 1.08 ± 0.39 in favor of the animals treated with 5% casein, but this ratio is not significantly different from 1.

II. INFLUENCE OF DIETARY PROTEIN ON ADRENAL WEIGHT RESPONSE TO LAP

Material and Methods.—Two groups of rats whose body weight ranged between 90–110 gm. were adapted to a 10% casein diet (diet 2) for a period of ten days. Group I was then hypophysectomized and was given LAP in two subcutaneous injections of 0.2 ml. per day, increasing progressively from 2 mg. on the first day, to 8 mg. on the seventh. Group III was maintained intact and received the same treatment as group I. Two other similar groups

TABLE 2. INFLUENCE OF DIET ON ADRENAL ASCORBIC ACID RESPONSE TO LAP IN HYPOPHYSECTOMIZED RATS

Group	Treatment	LAP dose (/100 gm. B.W.)	Ascorbic acid depletion
I	5% Casein	3.5	21*
		7.0	111
		14.0	156
II	30% Casein	3.5	50
		7.0	73
		14.0	145

* Each value represents the average of 2 animals.

(II and IV) of rats were adapted for ten days to a 30% casein diet and were treated as groups I and III respectively. All animals received a 5% glucose solution as drinking fluid after the hypophysectomy. At the end of seven days the animals were killed and the completeness of the hypophysectomy checked. The weight of the adrenals was determined after fixation in formalin. The differences in final body weight due to the diets required an exact statistical analysis of the results. It is customary to express the adrenal weight per 100 gm. of body weight as obtained by straight division of the latter by

TABLE 3. VARIANCE ANALYSIS OF ASCORBIC ACID DEPLETION TEST

Variance due to	Sums of squares	n	Variance	Z	P
Samples	126.75	1	126.75		
Slope	26680.50	1	26680.50	1.12	<0.05
Departure from parallelism	861.12	1	861.12		
Curvature of combined curve	4.17	1	4.17		
Opposed curvature of separate curves	1441.50	1	1441.50		
Repetitions	6674.16	1	6674.16	0.428	>0.05
Error	11320.79	4	2830.20		

the former. Korenchevsky (1942) showed that this relative value diminishes with increasing size of the animal. We therefore decided to correct for differences in the average body weights of the various groups by employing a body-adrenal weight regression line obtained through covariance analysis (Bliss and Marks, 1939, 1939a).

RESULTS

As may be seen from table 4, in the hypophysectomized rats there is no significant difference between the average adrenal weights of groups I (10% casein) and II (30% casein) either as absolute weights or corrected for body weight differences ($t=0.956$, $P\simeq 0.3$ and $t=1.806$, $P\simeq 0.1$ respectively). On the other hand, among the intact animals there is a significant difference between the average adrenal weights of groups III (10% casein) and IV (30% casein) expressed either way. (For absolute weights $t=3.986$, $P<0.01$ and for corrected weights $t=3.750$, $P<0.01$).

TABLE 4. INFLUENCE OF DIET ON THE ADRENAL WEIGHT RESPONSE TO LAP IN HYPOPHYSECTOMIZED AND INTACT RATS

Group	No. of animals	Treatment	Body weights		Adrenal weight	
			Initial	Final	mg.	Corrected for B.W.#
I	6	Hypo-ect* 10% casein + LAP	117.5 ± 5.9†	103 ± 16.3†	18.3 ± 0.62‡	18.6 ± 0.81
II	6	Hypo-ect 30% casein + LAP	120 ± 5.2	116 ± 17.3	20.0 ± 1.02	19.7 ± 0.81
III	8	10% casein + LAP	116 ± 3.2	130 ± 12.7	23.8 ± 1.02	24.1 ± 0.96
IV	8	30% casein + LAP	120 ± 2.1	148 ± 6.6	29.9 ± 1.15	29.2 ± 0.96

* Hypo-ect means hypophysectomy
† Standard deviation.
‡ Standard error.
# The correction was made by covariance analysis (Bliss and Marks, 1939, 1939a).

Incidentally, we should like to mention that even on very low protein diets, stress can increase the pituitary ACTH secretion to some extent. This is revealed by the changes in ascorbic acid concentration in the left adrenal of the hypophysectomized rats, that is, the gland removed before LAP injection. It is observed that the adrenal ascorbic acid concentration was consistently and significantly lower on 5% than on 30% casein diets. This fact suggests that the animals on 5% casein secreted an increased amount of ACTH previous to the hypophysectomy. Here the stress effect of the protein deficient diet presumably outweighed its power to inhibit corticotrophin secretion.

TABLE 5. INFLUENCE OF DIETARY PROTEIN ON ADRENAL ASCORBIC ACID CONCENTRATION

Tests	Adrenal ascorbic acid (mg./100 gm)*	
	5% casein	30% casein
1	349 ± 14.1† (7)	432 ± 16.7 (8)
2	352 ± 7.8 (6)	424 ± 26.2 (7)
3	448 ± 4.6 (6)	586 ± 41.7 (6)
4	454 ± 7.0 (6)	526 ± 11.8 (8)

* Control gland removed before injection.
† Standard errors.
Figures between brackets indicate number of animals.

Table 5 summarizes pertinent data obtained in three preliminary experiments (not reported here in detail) as well as in the ascorbic acid depletion test described above.

DISCUSSION

It is apparent that among intact animals, the adrenal enlarging activity of LAP is increased by a high-protein diet, but this is not so in hypophysectomized rats. The latter was observed in both ascorbic acid depletion and adrenal enlargement tests. Our results seem to exclude the possibility of an extra-hypophyseal activation of corticotrophic action by protein. But the possibility still remains that in intact animals under stress, the high-protein diet stimulates the elaboration by the pituitary of corticotrophic material. LAP could

also stimulate the hypophysis of intact animals through stress caused by its toxic components. It should be noted that in spite of LAP treatment, the hypophysectomized animals in experiment II did not gain weight during the week following operation, indicating inadequate food intake. The animals treated with 10% casein lost weight, while those on 30%, roughly maintained their body weights.

The possibility also exists that although protein does not activate LAP directly, it facilitates the formation of active corticotrophin from other LAP-constituents (precursors?) in the pituitary itself.

SUMMARY

Contrary to what is observed in intact rats, after hypophysectomy the corticotrophic action of lyophilized anterior pituitary (LAP) is not influenced by the dietary protein. This was shown both by the adrenal ascorbic-acid-depletion test and by the adrenal weight response of hypophysectomized rats given low and high-protein diets respectively. It appears that the ability of high-protein diets to increase the corticotrophic effect (of stress or LAP) is dependent upon the integrity of the hypophysis.

ACKNOWLEDGMENTS

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EFFECTS OF A HIGH PROTEIN DIET ON THE ANEMIA INDUCED BY HYPOPHYSECTOMY IN ADULT FEMALE RATS, INCLUDING FURTHER DETAILS ON POST-HYPOPHYSECTOMY ANEMIA[1]

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HYPOPHYSECTOMY induces a profound anemia in the rat (Stewart, Greep, and Meyer, 1935; Meyer, Stewart, Thewlis, and Rusch, 1937; Vollmer, Gordon, Levenstein, and Charipper, 1939; Crafts, 1941). Injections of thyroxine (Meyer, Thewlis, and Rusch, 1940; Vollmer, Gordon, and Charipper, 1942; Crafts 1946a), a combination of thyroxine, iron, and copper (Crafts, 1946a), and of androgen (Vollmer and Gordon, 1941; Vollmer, Gordon, and Charipper, 1942; Crafts, 1946b) prevented all aspects of this anemia except for the decrease in hemoglobin values. Since the above treatments did not produce a normal hemoglobin, the metabolism of iron was investigated following hypophysectomy. A decrease in volume of gastric juice and a decrease in serum iron was found following hypophysectomy in adult female rats, but the concentration of storage iron was increased (Crafts and Walker, 1947a and b). Utilizing the isotope technique, it was found that there were differences in the metabolism of iron between normal and hypophysectomized rats but that these differences were not of a degree to account for the lack of hemoglobin formation (Crafts, Ross, and Walker, in press).

The present investigation was done in an attempt to answer the question whether the decrease in hemoglobin formation following hypophysectomy is due to a lack of protein to combine with the available iron. Fifty-seven hypophysectomized adult female rats were divided into the following five groups and given the following treatments:—(1) no treatment, (2) a high protein diet, (3) a high protein diet plus daily injections of thyroxine, (4) a high protein diet plus daily injections of testosterone propionate, and (5) a high protein diet plus daily injections of both thyroxine and testosterone propionate.

METHODS

Adult female rats of the Long-Evans strain were used in these experiments. The average body weight of these animals was 196 (S.D. 16.0) gm.

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[2] With the technical assistance of Miss Shirley A. Raymond.

Hypophysectomy was performed from the parapharyngeal approach and the completeness of the operation was checked by serial sections of the hypophyseal capsule.

Because no studies were contemplated on the white blood cells, blood for these studies was obtained by clipping the tail. Absolutely no "milking" of the tail was performed. Five to six drops of free flowing blood were placed in a small vial containing powdered heparin.

Erythrocyte counts were made in duplicate using standard U. S. certified pipettes and the improved Neubauer counting chamber. Hemoglobin determinations were made on an Evelyn photoelectric colorimeter. Van Allen tubes were used for hematocrit determinations. Mean corpuscular diameter (M.C.D.) was measured in a Haden-Hausser erythrocytometer using cover slip smears stained with Wright's stain and mounted in physiological saline solution. Mean corpuscular volume in cubic microns (M.C.V.), mean corpuscular hemoglobin in micromicrograms (M.C.H.), and mean corpuscular hemoglobin concentration in per cent (M.C.H.C.) were determined by the formulae cited below.[3]

The high protein diet was obtained by combining the standard diet (Rockland Farms complete rat diet) with powdered casein in equal quantities. This produced a diet containing 62.5% protein. Fresh lettuce was given weekly.

PROCEDURE AND RESULTS

Table 1 shows the average figures obtained for the 57 rats used in these experiments before any treatment. The erythrocyte count averaged 7.83 million cells per cu. mm.; hematocrit, 43.1%; hemoglobin, 14.0 gm. per 100 cc.; M.C.D., 5.63 micra; M.C.V., 55.2 cu. microns; M.C.H., 18.0 micromicrograms; and the M.C.H.C., 32.5%. These figures for the erythrocyte count, hemoglobin, and the diameter closely agree with figures previously published from this laboratory. The hematocrit (43.1%) is slightly lower than the average of 45.05% found by Gardner (1947) in summarizing values in the literature. Her average, however, includes both male and female rats.

Hypophysectomy

Hypophysectomy induced an anemia. Much data have been published on the blood picture during the first 40 days after hypophysectomy. Accordingly, these rats were studied from the 40th to the 95th day after removal of the hypophysis. Hypophysectomy induced a decrease in the erythrocyte count (Table 1, Fig. 1) to between 5 and 6 million cells per cu. mm. The counts reached that

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[3] $\text{M.C.V.} = \dfrac{\text{vol. packed red cells, cc. per 1000 cc.}}{\text{red cell count, millions per c.mm.}}$

$\text{M.C.H.} = \dfrac{\text{hemoglobin, gm. per 1000 cc.}}{\text{red cell count, millions per c.mm.}}$

$\text{M.C.H.C.} = \dfrac{\text{hemoglobin, gm. per 100 cc.} \times 100}{\text{vol. packed red cells, cc. per 100 cc.}}$

TABLE 1. EFFECTS OF HYPOPHYSECTOMY, TREATMENT WITH A HIGH PROTEIN DIET ALONE, AND TREATMENT WITH A HIGH PROTEIN DIET PLUS INJECTIONS OF THYROXINE AND TESTOSTERONE PROPIONATE ON THE BLOOD PICTURE OF HYPOPHYSECTOMIZED ADULT FEMALE RATS

DAY = Days of treatment; BW = Body weight; TRC = Total red cell count in millions per cu. mm.; HEMAT = Hematocrit in percent; HB = Hemoglobin in gm. per 100 cc; M.C.D. = Mean corpuscular diameter in micra; M.C.V. = Mean corpuscular volume in cu. microns; M.C.H. = Mean corpuscular hemoglobin in micromicrograms; M.C.H.C. = Mean corpuscular hemoglobin concentration in percent; ± = Standard deviation.

Normal control figures on all rats before hypophysectomy—57 rats.

DAY	BW	TRC	HEMAT	HB	M.C.D.	M.C.V.	M.C.H.	M.C.H.C.
0	196	7.83	43.1	14.0	5.63	55.2	18.0	32.5
	±16	±0.45	±1.7	±0.7	±0.21	±2.5	±0.9	±1.4

Hypophysectomized—no treatment—5 rats.

DAY	BW	TRC	HEMAT	HB	M.C.D.	M.C.V.	M.C.H.	M.C.H.C.
40	153	6.20	30.5	10.2	5.52	49.3	16.6	33.6
	±6	±0.35	±0.9	±0.4	±0.11	±2.1	±0.8	±0.4
50	159	5.95	30.4	10.3	5.54	51.5	17.4	33.8
	±15	±0.53	±2.1	±0.5	±0.13	±2.3	±1.3	±1.4
60	153	5.64	29.5	9.7	5.75	53.0	17.4	33.0
	±6	±0.43	±1.7	±0.5	±0.19	±5.7	±1.4	±2.0
75	140	5.19	28.7	9.4	5.55	55.3	18.1	32.7
	±4	±0.15	±1.0	±0.3	±0.17	±1.8	±0.3	±0.6
95	132	5.99	29.8	9.9	5.89	50.8	16.5	32.6
	±14	±0.37	±1.3	±1.0	±0.23	±0.9	±0.8	±1.8

Hypophysectomized and treated with a high protein diet—8 rats.

DAY	BW	TRC	HEMAT	HB	M.C.D.	M.C.V.	M.C.H.	M.C.H.C.
0	194	7.79	43.7	13.9	5.81	56.1	17.9	31.9
	±19	±0.36	±1.0	±0.4	±0.24	±1.9	±0.7	±0.7
10	178	8.29	45.4	15.1	5.56	54.8	18.2	33.2
	±15	±0.64	±4.1	±1.3	±0.08	±1.3	±0.8	±1.3
20	173	8.24	45.3	14.5	5.60	55.1	17.6	31.8
	±16	±0.94	±4.7	±1.2	±0.09	±2.1	±0.8	±0.9
30	178	7.14	37.9	12.5	5.67	53.2	17.6	33.2
	±11	±0.84	±3.7	±1.1	±0.08	±4.1	±0.9	±1.9
40	183	7.05	35.9	12.1	5.61	51.0	17.2	33.9
	±10	±0.24	±2.9	±0.5	±0.11	±4.7	±0.8	±2.4
50	169	6.82	36.8	12.1	5.66	53.8	17.7	32.9
	±14	±0.39	±0.8	±0.4	±0.06	±2.2	±0.9	±0.9

Hypophysectomized and treated with a high protein diet plus daily injections of 0.005 mg. of thyroxine. On day 30 the dose of thyroxine was changed to 0.015 mg. per day—14 rats.

DAY	BW	TRC	HEMAT	HB	M.C.D.	M.C.V.	M.C.H.	M.C.H.C.
0	194	7.90	43.2	13.7	5.65	54.5	17.4	31.8
	±23	±0.53	±1.9	±0.9	±0.06	±2.1	±0.6	±0.9
10	179	7.81	42.3	13.8	5.58	53.8	17.8	32.8
	±21	±0.46	±1.7	±0.5	±0.13	±2.5	±0.8	±1.0
20	172	7.55	39.7	12.9	5.60	52.5	17.1	32.2
	±20	±0.42	±2.0	±1.4	±0.14	±2.5	±1.7	±3.0
30	164	7.33	37.6	12.3	5.61	51.2	16.7	32.7
	±23	±0.69	±3.0	±1.0	±0.13	±2.3	±0.9	±0.9
40	154	7.65	39.6	12.8	5.70	52.2	16.8	32.2
	±24	±0.81	±2.6	±0.9	±0.13	±3.3	±1.2	±0.8
50	146	7.56	41.1	12.8	5.63	54.4	16.9	31.0
	±21	±0.56	±3.0	±0.9	±0.08	±2.8	±0.9	±0.7
60	149	7.65	40.1	12.7	5.80	52.6	16.6	31.6
	±15	±0.47	±1.8	±0.7	±0.15	±2.7	±1.0	±1.2

TABLE 1—*Cont.*

Hypophysectomized and treated with a high protein diet plus daily injections of 2.0 mg. of testosterone propionate—18 rats.

DAY	BW	TRC	HEMAT	HB	M.C.D.	M.C.V.	M.C.H.	M.C.H.C.
0	195	7.67	42.6	13.9	5.72	55.6	18.3	32.6
	±11	±0.41	±1.8	±0.4	±0.13	±2.6	±0.9	±1.6
10	175	7.98	44.3	14.5	5.71	56.4	18.2	32.3
	±16	±0.88	±4.3	±1.4	±0.12	±4.0	±1.3	±1.0
20	172	7.68	42.3	13.7	5.63	55.1	17.9	32.4
	±10	±0.75	±4.1	±1.4	±0.07	±2.4	±0.7	±1.1
30	180	7.36	39.6	12.8	5.75	53.7	17.5	32.2
	±10	±0.24	±1.0	±0.4	±0.13	±2.0	±0.6	±1.2
40	176	7.42	38.4	12.4	5.65	52.1	16.8	32.2
	±9	±0.63	±2.0	±0.5	±0.11	±3.7	±1.6	±1.6
50	176	8.31	39.9	12.9	—	48.4	15.5	32.4
	±11	±0.33	±0.7	±0.4	—	±2.3	±0.8	±1.0

Hypophysectomized and treated with a high protein diet plus daily injections of 0.005 mg. of thyroxine and 1.0 mg. of testosterone propionate. On day 40 all treatment was stopped—12 rats.

DAY	BW	TRC	HEMAT	HB	M.C.D.	M.C.V.	M.C.H.	M.C.H.C.
0	201	7.98	43.3	14.5	5.35	54.2	18.2	33.5
	±16	±0.43	±1.8	±0.6	±0.15	±2.7	±0.9	±0.9
10	181	8.02	43.0	14.1	5.85	53.9	17.7	32.9
	±11	±0.41	±1.4	±0.7	±0.26	±2.2	±0.7	±1.1
20	186	8.43	44.7	14.6	5.56	53.0	17.3	32.6
	±12	±0.23	±1.7	±0.6	±0.24	±2.4	±0.9	±0.7
30	178	8.48	44.2	14.1	5.54	52.0	16.7	32.0
	±11	±0.34	±1.4	±0.7	±0.25	±2.6	±1.1	±0.8
40	173	8.42	44.0	13.9	5.69	52.3	16.5	31.6
	±19	±0.59	±2.9	±0.8	±0.10	±2.6	±0.7	±0.7
				All treatment stopped				
10	162	8.48	42.1	13.3	5.85	50.0	15.7	31.5
	±11	±0.65	±2.1	±0.8	±0.08	±2.4	±0.7	±0.8
20	156	7.66	38.4	12.2	5.90	50.2	15.9	31.7
	±16	±0.46	±2.6	±0.6	±0.06	±2.4	±0.6	±1.9
30	152	7.24	34.0	10.7	5.51	47.0	14.8	31.6
	±14	±0.16	±1.8	±0.5	±0.09	±1.7	±0.6	±0.7

level at 50 days and remained at that level for another 45 days. This is a 29.8% decrease. Hematocrit values (Table 1, Fig. 2) decreased to 30.5% on the 40th day after hypophysectomy and remained between 28.7% and 30.5% for the entire time of observation, a 32.6% decrease. Hemoglobin determinations (Table 1, Fig. 3) showed a decrease to 10.2 gm. per 100 cc. on the 40th day after removal of the hypophysis and remained between 9.0 and 10.0 gm. for the remaining 55 days of observation. This is a 31.4% decrease.

The mean corpuscular diameter (M.C.D. Table 1) on the 40th day after hypophysectomy averaged 5.52 micra, a slight decrease from the control average of 5.63 micra. During the subsequent 55 days of observations, while the erythrocyte count, hemoglobin, and hematocrit reached a plateau, the M.C.D. fluctuated. This change is slight compared to the data previously reported (Crafts, 1946a) indicating a more definite microcytosis. This previous work, however, was based on studies at 30 and 40 days after hypophysectomy and on actual measurements with an ocular micrometer.

In spite of the slight decrease in diameter, the mean corpuscular

volume (M.C.V. Table 1) decreased to 49.3 cu. microns (Control 55.2) 40 days after hypophysectomy. Observations during the subsequent 55 days showed a gradual increase to a normal value of 55.3 cu. microns on the 75th day and a drop to 50.8 on the 95th day after removal of the hypophysis.

The mean corpuscular hemoglobin (M.C.H. Table 1) followed the same course as the mean corpuscular volume. At 40 days the M.C.H.

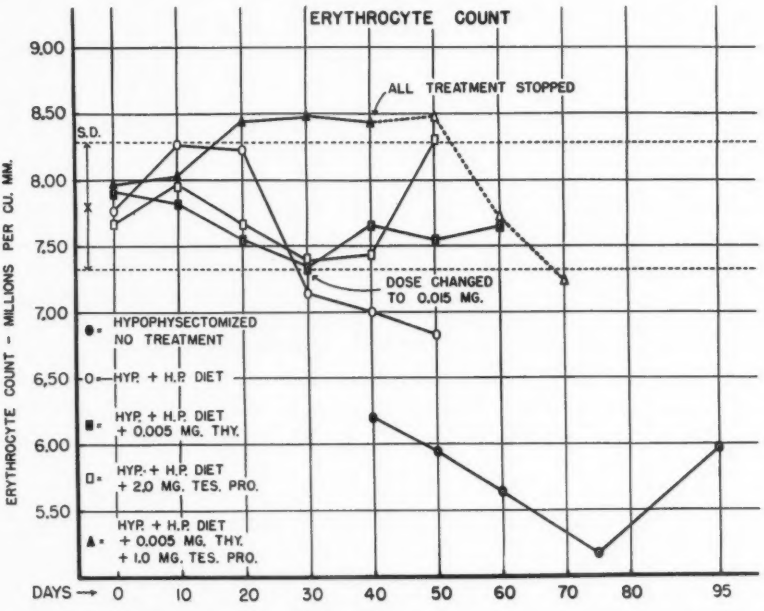

FIG. 1. The effects of a high protein diet, with or without thyroxine or testosterone propionate therapy, on the erythrocyte count of hypophysectomized adult female rats. X − S.D. = mean and standard deviation of all rats before treatment.

was 16.6 micromicrograms (Controls 18.0). The values gradually increased to a normal figure of 18.1 micromicrograms and then dropped to 16.5 on the 95th day after hypophysectomy.

The mean corpuscular hemoglobin concentrations (M.C.H.C. Table 1) showed no change from normal values.

In summary, hypophysectomy induced a profound anemia. The erythrocyte count, hematocrit, and hemoglobin decreased rapidly for 40 days after which no further decrease occurred. From the 40th to the 95th days after hypophysectomy the mean corpuscular diameter, volume, and hemoglobin fluctuated, at one time showing a decrease and at another time showing normal values. The mean corpuscular hemoglobin concentration did not change, indicating that regardless of size of the erythrocytes, each carried a normal concen-

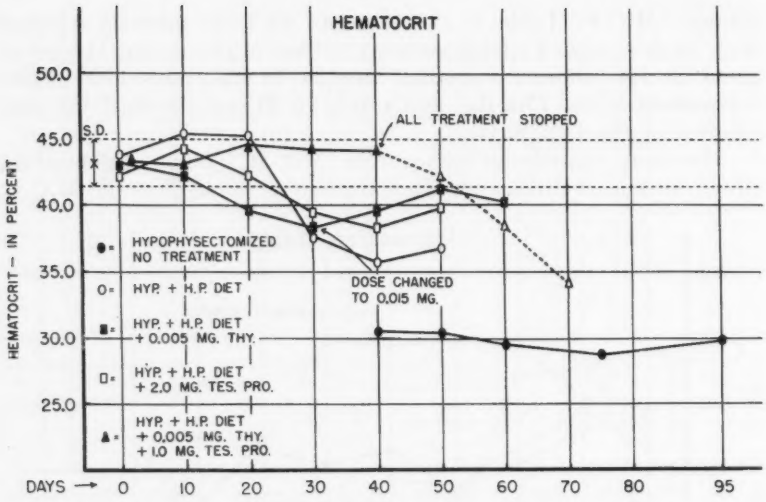
HEMATOCRIT
HEMATOCRIT – IN PERCENT
50.0
45.0
40.0
35.0
30.0
25.0
S.D.
X
ALL TREATMENT STOPPED
DOSE CHANGED TO 0.015 MG.
●= HYPOPHYSECTOMIZED NO TREATMENT
O= HYP. + H.P. DIET
■= HYP. + H.P. DIET + 0.005 MG. THY.
□= HYP. + H.P. DIET + 2.0 MG. TES. PRO.
▲= HYP. + H.P. DIET + 0.005 MG. THY. + 1.0 MG. TES. PRO.
DAYS → 0 10 20 30 40 50 60 70 80 95

FIG. 2. The effects of a high protein diet, with or without thyroxine or testosterone propionate therapy, on the hematocrit values of hypophysectomized adult female rats. X – S.D. = mean and standard deviation of all rats before treatment.

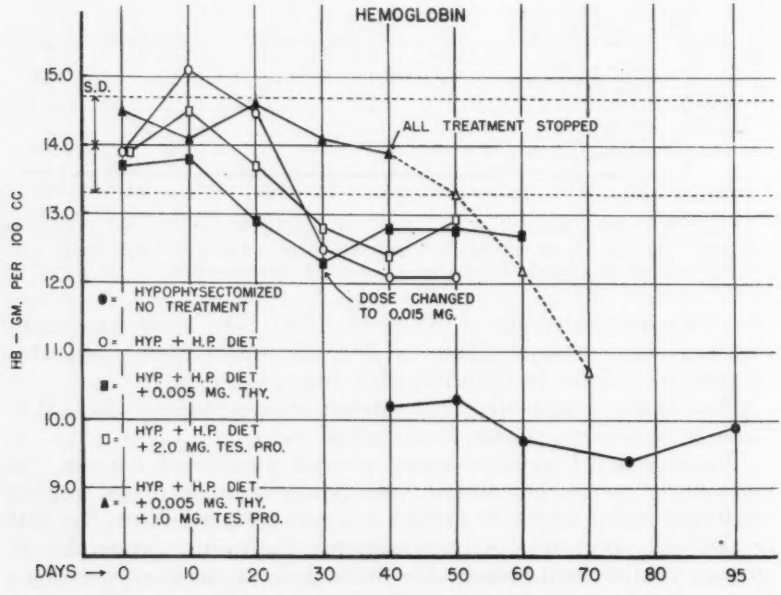
HEMOGLOBIN
HB – GM. PER 100 CC
15.0
14.0
13.0
12.0
11.0
10.0
9.0
S.D.
X
ALL TREATMENT STOPPED
DOSE CHANGED TO 0.015 MG.
●= HYPOPHYSECTOMIZED NO TREATMENT
O= HYP. + H.P. DIET
■= HYP. + H.P. DIET + 0.005 MG. THY.
□= HYP. + H.P. DIET + 2.0 MG. TES. PRO.
▲= HYP. + H.P. DIET + 0.005 MG. THY. + 1.0 MG. TES. PRO.
DAYS → 0 10 20 30 40 50 60 70 80 95

FIG. 3. The effects of a high protein diet, with or without thyroxine or testosterone propionate therapy, on the hemoglobin values of hypophysectomized adult female rats. X – S.D. = mean and standard deviation of all rats before treatment.

tration of hemoglobin. Any decrease in mean corpuscular hemoglobin, therefore, was due to the decrease in volume of the erythrocytes. The hypophysectomized rat finally showed a profound anemia which may be considered as a normocytic normochromic type or a slight microcytic normochromic type. These findings agree with the many reports in the literature for humans with hypopituitary conditions. Some patients show a normocytic normochromic type of anemia while others show a microcytic normochromic type. A single patient may vary from one type to the other at different times just as is indicated in these data for the rat.

High Protein Diet

Fourteen rats were hypophysectomized and immediately placed on a high protein diet. No further treatment was given. Blood was studied at 10 day intervals for 50 days. Detailed results are given in Table 1.

The erythrocyte count (Fig. 1) for this group of rats before hypophysectomy averaged 7.79 million cells per cu. mm. On the 10th day the count had increased to 8.29 million cells, remained at that level on the 20th day, and then gradually decreased to 6.82 million cells 50 days after hypophysectomy. This is a 12.9% decrease.

Hematocrit values followed the same curve (Fig. 2), increasing from the normal level of 43.7% to 45.4 and 45.3% on days 10 and 20 respectively, and then gradually decreasing to 35.9% on day 40 and 36.8% on day 50. This is a 15.8% decrease.

The hemoglobin (Fig. 3) before treatment averaged 13.9 gm. per 100 cc., increased to 15.1 gm. on day 10, and then gradually decreased to 12.1 gm. on days 40 and 50, a 12.9% decrease.

The changes in mean corpuscular diameter, volume, and hemoglobin were slight, probably insignificant decreases, while the M.C.H.C. remained normal.

Summarizing, a high protein diet did not prevent an anemia from developing following the removal of the hypophysis. The anemia, however, was not as severe as is found after hypophysectomy and no treatment. Erythrocyte count, hematocrit, and hemoglobin decreased 13, 16, and 13% respectively, while after hypophysectomy alone there were 30, 33, and 31% decreases in these figures.

High Protein Diet + Thyroxine

Thyroxine therapy has been found to alleviate many of the signs of the anemia induced by hypophysectomy. In addition, moderate doses of thyroxine are known to stimulate the utilization of proteins. Accordingly, 14 hypophysectomized rats were injected with daily doses of 0.005 mg. of thyroxine[4] in addition to the high protein diet. Treatment was started the day after hypophysectomy. The dose of

[4] The thyroxine was very kindly furnished by Hoffmann-La Roche, Inc.

thyroxine was elevated to 0.015 mg. on the 30th day. Blood was studied at 10 day intervals for 60 days. Detailed results are shown in Table 1.

The erythrocyte count (Fig. 1) in this group of rats before hypophysectomy averaged 7.90 million cells per cu. mm. There was a decrease to 7.33 million cells by the 30th day but an increase to 7.65 million cells after 30 days of treatment at the high dose. This is only a 3.2% decrease.

The hematocrit (Fig. 2) averaged 43.2% before hypophysectomy. Thirty days after removal of the hypophysis the hematocrit averaged 37.6%, and with the higher dose of thyroxine increased to 40.1% on the 60th day. This is a 7.2% decrease.

Hemoglobin values (Fig. 3) started at an average of 13.7 gm. per 100 cc. and decreased to 12.7 gm. by the 60th day, a 7.7% decrease.

The M.C.V. and M.C.H. showed a slight decrease while the M.C.D. and M.C.H.C. showed no changes.

Summarizing, hypophysectomized rats fed a high protein diet and treated with daily injections of thyroxine for 60 days showed a 3% drop in erythrocyte count, a 7% drop in hematocrit, and an 8% drop in hemoglobin. These decreases are small when compared to a 30% erythrocyte count drop, a 33% hematocrit drop, and a 31% hemoglobin drop which followed hypophysectomy and no treatment.

High Protein Diet + Testosterone Propionate

Testosterone propionate has also been found to alleviate most of the signs of the anemia which is induced by removal of the hypophysis. This hormone is well known to be a stimulant to the utilization of protein in the normal animal and has been reported to have a similar action in the hypophysectomized rat (Kochakian, Moe, Hunter, and Stettner, 1947). Accordingly, 18 hypophysectomized rats were given a high protein diet plus daily injections of 2.0 mg. of testosterone propionate[5] for 50 days starting the day following the removal of the hypophysis. Blood was studied at 10 day intervals. Details are presented in Table 1.

Erythrocyte values (Fig. 1) started at an average of 7.67 million cells per cu. mm. before treatment. By the 50th day the count had increased to 8.31 million cells, a 7.8% increase rather than a decrease. Hematocrit values (Fig. 2) started at 42.6% and decreased to 39.9%, a 6.3% decrease. Hemoglobin values (Fig. 3) decreased from the normal average of 13.9 gm. per 100 cc. to 12.9 gm., a decrease of 7.2%.

The M.C.V. and M.C.H. decreased from 55.6 to 48.4 cu. microns (13.0% decrease) and from 18.3 to 15.5 micromicrograms (15.3% decrease) respectively. The M.C.D. and M.C.H.C. showed no significant changes.

In summary, testosterone propionate treatment, in addition to

[5] Testosterone propionate was very kindly furnished by Schering Corporation, Bloomfield, N. J.

the high protein diet, maintained a normal erythrocyte count. Hematocrit and hemoglobin values decreased only 6 and 7% respectively. There was a 13% decrease in M.C.V. and a corresponding 15% decrease in M.C.H.

High Protein Diet + Thyroxine + Testosterone Propionate

Twelve hypophysectomized rats were treated with daily injections of a combination of 0.005 mg. of thyroxine and 1.0 mg. of testosterone propionate in addition to the high protein diet. These rats were studied at 10 day intervals for 40 days. At this time, the 40th day, all treatment was stopped, the animals being placed on the regular diet. Details are presented in Table 1.

The erythrocyte count (Fig. 1) in this group of rats averaged 7.98 million cells per cu. mm. before treatment. The count increased to 8.42 million cells in 40 days of treatment, a 5.2% increase. After treatment was stopped, the count remained at this high level for 10 days and then decreased to 7.24 million cells 30 days after cessation of treatment.

Hematocrit determinations (Fig. 2) increased from 43.3% to 44.0% in 40 days of treatment. Although this is not a significant increase, it is the only treatment in this series of experiments in which no decrease occurred. After treatment was stopped, the hematocrit decreased to 34.0% in 30 days.

The hemoglobin average for this group of rats before treatment was slightly higher than that of the other groups (Fig. 3). This group started at 14.5 gm. per 100 cc. and decreased to 13.9 gm. in 40 days. This figure is a normal average for these rats, for the average of all 57 rats in this series before treatment was only 0.1 gm. higher, i.e. 14.0 (S.D. 0.7) gm. The decrease from 14.5 gm. to 13.9 gm. is a 4.1% drop. After treatment was stopped, the hemoglobin decreased to 10.7 gm. in 30 days, a figure typical of the hypophysectomized animal with no treatment.

The M.C.D. increased slightly and the M.C.V., M.C.H., and the M.C.H.C. declined slightly. After treatment was stopped, the M.C.D. declined, the M.C.V. and M.C.H. decreased even lower, and the M.C.H.C. remained the same.

In summary, hypophysectomized rats, when treated with a high protein diet plus injections of both thyroxine and testosterone propionate, exhibited no anemia. When treatment was stopped, the erythrocyte count, hematocrit, and hemoglobin values decreased. During treatment, the erythrocyte and hematocrit values were increased while the hemoglobin was merely maintained at normal levels. In addition, the M.C.V. declined slightly. As a result the hemoglobin per cell (M.C.H.) decreased slightly and the concentration of hemoglobin in each cell (M.C.H.C.) also decreased slightly. The latter figure, however, is well within the normal range. After treatment was stopped, these effects were accentuated.

DISCUSSION

Adult rats develop an anemia following the removal of the hypophysis. The erythrocyte count exhibits a 30% decrease during the first 40 days after hypophysectomy; the hematocrit, a 33% decrease; and the hemoglobin, a 31% decrease. From the 40th to the 95th day after hypophysectomy the erythrocyte count, hematocrit, and hemoglobin reach a plateau showing no further decreases. The erythrocytes have a decreased mean corpuscular volume and an accompanying decrease in mean corpuscular hemoglobin, although these decreases are small. Each cell, however, carries a normal concentration of hemoglobin which shows that the decrease in mean corpuscular hemoglobin is due to the decrease in size of the erythrocytes. This anemia fluctuates between a normocytic normochromic type and a microcytic normochromic type. The bone marrows are hypoplastic and exhibit a decrease in number of erythroid elements (Crafts, 1946a and b). This anemia is similar to that observed in human hypopituitary patients as reported often in the literature. (See review article by Daughaday, Williams, and Daland, 1948.)

Injections of thyroxine or androgens have been shown to prevent all aspects of this anemia except for the fall in hemoglobin. The data in this report show that the fall in hemoglobin level, in addition to other aspects of the anemia, can be prevented when a high protein diet is added to a combined thyroxine and androgen therapy.

Is the anemia which occurs following the removal of the hypophysis actually due to faulty protein metabolism? Hypophysectomy is well known to interfere with protein metabolism. In addition, Cartwright (1947), in a review article, cites many references showing that an anemia is induced by decreasing the protein content of the diet below normal, and that the formation of hemoglobin is enhanced by a return to a normal or high protein diet. Levin (1943) has shown that the hypophysectomized animal is not able to utilize endogenous protein. On the other hand, if faulty protein metabolism is the partial or complete cause for the anemia which hypophysectomy induces, growth hormone injections should be a logical therapy. As yet, this has not proved to be so. Meyer, Stewart, Thewlis, and Rusch (1937) injected hypophysectomized rats with growth hormone (Antuitrin S—Parke Davis) in the dose of 1 cc. three times a week. They obtained a marked response in reticulocytes but the erythrocyte count and hemoglobin did not rise. When they combined the growth hormone with thyroxine, a good response was obtained. Growth hormone alone, in the doses given and the preparation used, was not effective. No reports have been published on the effects of the purified growth hormone preparations on this anemia.

The anemia induced by hypophysectomy can be prevented by a high protein diet in combination with thyroxine and androgen therapy. The beneficial effects of thyroxine and testosterone propi-

onate may be due to their influence on general metabolism or to their influence on protein metabolism. It is safe to conclude that the anemia that is induced by removal of the hypophysis is due to faulty metabolism and that there is no necessity for a "hemopoietic hormone" secreted by the hypophysis as proposed by Flaks, Himmel, and Zlotnik (1937–1938).

SUMMARY

Hypophysectomy induces an anemia in adult rats which has the following characteristics: (1) a 30% decrease in erythrocyte count, (2) a 33% decrease in hematocrit, (3) a 31% decrease in hemoglobin, (4) a slight decrease in mean corpuscular volume which caused (5) a slight decrease in mean corpuscular hemoglobin, (6) a normal mean corpuscular hemoglobin concentration, and (7) a hypoplasia of the bone marrow with a decrease in percentage of erythroid elements.

Thyroxine or androgen therapy prevent all manifestations of this anemia except for the fall in hemoglobin. This experiment was conducted to determine whether this lack of hemoglobin formation was due to a lack of available protein. 57 hypophysectomized adult female rats were divided into 5 groups: (1) no treatment, (2) fed a high protein diet, (3) a high protein diet + thyroxine, (4) a high protein diet + androgen, and (5) a high protein diet + thyroxine + androgen.

Best results were obtained in the last group with a high protein diet + 0.005 mg. of thyroxine per day + 1.0 mg. of testosterone propionate per day. This therapy maintained the hematocrit and hemoglobin at normal levels while increasing the erythrocyte count. The anemia induced by hypophysectomy can be prevented by a high protein diet in combination with thyroxine and androgen therapy.

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STUDIES ON THE NATURE OF THE PROTEIN CATABOLIC RESPONSE TO ADRENAL CORTICAL EXTRACT. ACCENTUATION BY INSULIN HYPOGLYCEMIA

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IN PREVIOUS REPORTS (Engel, Schiller and Pentz, 1949, Bondy, Engel, and Farrar, 1949) data were presented to indicate that the adrenal cortex influenced nitrogen metabolism largely at the level of protein breakdown and that this effect on protein catabolism was conditioned by the internal metabolic environment of the animal at the time of treatment with hormone. It was suggested that the levels of carbohydrate stores in the body and the availability of sources of new carbohydrate other than tissue protein were factors in determining the occurrence or magnitude of protein catabolism after cortical hormone treatment. A possible synergistic role of non-specific stress in the protein catabolic response to adrenal cortical extract (A.C.E.) was also considered.

The present study is an attempt to elucidate further the role of the need for gluconeogenesis in determining the protein catabolic response to A.C.E. Insulin hypoglycemia was used as a method for increasing carbohydrate requirements and hence the need for gluconeogenesis in the fasting animal. It was found that insulin alone had no immediate effect on the rate of urea accumulation in nephrectomized rats but caused a delayed increase whereas insulin plus A.C.E. resulted in an immediate and striking acceleration of urea formation which was 3–4 times as great as that after A.C.E. alone. Prevention of hypoglycemia by glucose obliterated the increase in nitrogen metabolism.

MATERIALS AND METHODS

These were essentially the same as in previous reports (Engel, Pentz, and Engel, 1948, Engel, Schiller and Pentz, 1949, Bondy, Engel and Farrar, 1949) where full details may be obtained. Use was made of the rate of urea formation in the fasted nephrectomized rat, expressed as mg. urea N/100 grams body weight/hour, as a measure of protein catabolism. The animals

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[2] We are indebted to Dr. D. J. Ingle and the Upjohn Company for generous supplies of Adrenal Cortex Extract.

were nephrectomized 16 hours before the experimental procedure was begun and rates of urea formation studied for successive 3-hour periods. In all cases the first three-hour period was used as a control before test materials other than A.C.E. were injected. Blood urea nitrogen was determined by the method of Archibald, Ortiz, Stroh and Bronner (1945) and blood glucose by the method of Nelson (1944). The xanthydrol method for urea (Engel and Engel, 1947) previously used in these studies was not employed because of difficulty in obtaining an adequate grade of xanthydrol and subsequent highly irregular results from that material available. Squibb's insulin, diluted with saline and Upjohn's adrenal cortex extract were used throughout and were injected subcutaneously.

TABLE 1. INFLUENCE OF A.C.E. AND INSULIN ON THE UREA PRODUCTION OF NEPHRECTOMIZED RATS

Procedure	No. of rats	*Urea Nitrogen—Mg N per 100 Gm Body Weight per hour*						
		0–3 hours	3–6 hours	Change	P	6–9 hours	Change	P
A.C.E. (1.0 ml per 100 gms)*	10	1.5±0.24‡	2.4±0.25	+0.9±0.28	<0.01	—	—	—
Insulin (0.08 unit per 100 gms)†	13	2.3±0.21	3.0±0.35	+0.7±0.41	0.1	4.3±0.28	+2.0±0.23	<0.01
Insulin (0.5 unit per 100 gms)†	11	2.2±0.21	2.5±0.31	+0.3±0.35	>0.4	—	—	—
A.C.E. (1.0 ml per 100 gms)* Insulin (0.08 unit per 100 gms)†	7	2.9±0.39	5.6±0.68	+2.7±0.46	<0.01	—	—	—
A.C.E. (1.0 ml per 100 gms)* Insulin (0.25 unit per 100 gms)†	7	1.8±0.23	4.9±0.46	+3.1±0.46	<0.01	—	—	—
A.C.E. (1.0 ml per 100 gms)* Insulin (0.5 unit per 100 gms)†	10	2.0±0.31	5.1±0.27	+3.1±0.41	<0.01	—	—	—

* 0.5 ml A.C.E./100 gms at 0 and 1 hours.
† Injected at 3rd hour
‡ Standard Error.

RESULTS

Table 1 shows the effects of A.C.E., insulin, and A.C.E. plus insulin on the rates of urea formation during successive three hour periods. Table 2 records the blood sugar levels in these experiments. 0.5 ml. A.C.E. per 100 grams body weight was injected subcutaneously at 0 and 1 hours (16 and 17 hours after nephrectomy) and insulin at the third hour in doses of 0.5, 0.25 and 0.08 unit per 100 grams body weight. In agreement with previous observations (Engel, Pentz and Engel, 1948, Engel, Schiller and Pentz, 1949) the injection of 1.0 ml of A.C.E. in divided doses at 0 and 1 hours was followed by a significant increase in urea formation during the second 3 hour period, amounting to 0.9 ±0.28 mg. urea nitrogen per 100 grams body weight per hour. As shown previously and confirmed in Table 4, A.C.E. does not effect urea formation during the first three hours after its administration, but its effect is fully demonstrable 3–6 hours after injection. The blood sugar showed a small, but not significant increase between the 3rd and 6th hours. Insulin in doses of 0.08 and 0.5 unit per 100 grams body weight caused small but variable and hence statistically insignificant increases in urea formation during the

three hours following injection and significantly lowered the blood sugar. In the next three hour period (6–9 hours) a highly significant increase in urea formation occurred after 0.08 unit of insulin, injected at the 3rd hour. This amounted to 2.0 ±0.23 mg. N/100 grams/hour. It is probable that this delayed increase after insulin is related to endogenous adrenal cortical hormone production. The blood sugar had risen from 40 ±3.0 mg. per cent to 74 ±4.6 mg. per cent (Table 2). In three series of rats treated as before with 1.0 ml A.C.E./100 grams during the first 3 hours, injection of 0.5, 0.25 and 0.08 unit of insulin at the 3rd hour was followed by a very striking increase in the rate of urea formation in all cases during the 3–6 hour period. This increase was 3–4 times as great as that after A.C.E. alone. With 0.08

TABLE 2. MEAN BLOOD SUGAR LEVELS WITH STANDARD ERRORS AFTER A.C.E. AND INSULIN IN NEPHRECTOMIZED RATS

Procedure	Number of rats	Blood sugar in Mg %		
		3rd hour	6th hour	9th hour
A.C.E. (1.0 ml per 100 gms)*	10	125 ±2.7	144 ±8.4	—
Insulin (0.08 unit per 100 gms)†	13	106 ±3.3	40 ±3.0	74 ±4.6
Insulin (0.5 unit per 100 gms)† A.C.E. (1.0 ml per 100 gms)*†	11	126 ±4.6	41 ±8.8	—
Insulin (0.08 unit per 100 gms)† A.C.E. (1.0 ml per 100 gms)*†	7	97 ±2.9	40 ±9.2	—
Insulin (0.25 unit per 100 gms)† A.C.E. (1.0 ml per 100 gms)*†	7	101 ±9.1	37 ±6.4	—
Insulin (0.5 unit per 100 gms)†	10	100 ±8.7	42 ±4.5	—
Insulin (0.5 unit per 100 gms) + Glucose (500 mg per 100 gms)† A.C.E. (1.0 ml per 100 gms)*†	8	125 ±6.9	153 ±17.4	—
Insulin (0.5 unit per 100 gms) + Glucose (500 mg per 100 gms)†	7	105 ±7.1	195 ±30.6	
A.C.E. (1.0 ml per 100 gms)†	6	—	—	
A.C.E. (1.0 ml per 100 gms)†+ Insulin (0.08 unit per 100 gms)†	8	106 ±5.2	38 ±4.0	68 ±5.4

* 0.5 ml A.C.E./100 gms injected at 0 and 1 hours.
† Injected at 3rd hour.

unit of insulin it was not significantly greater than that occurring during the 6–9 hour interval following the same dose of insulin alone. The pretreatment with A.C.E. did not prevent or decrease the hypoglycemia following insulin (Table 2).

The data in Table 3 indicate that the accelerated rate of urea formation is due to the hypoglycemia rather than to the insulin per se. Prevention of the hypoglycemia by the injection of large amounts of glucose (500 mg. per 100 grams body weight) simultaneously with 0.5 unit of insulin completely prevented the change in urea nitrogen formation previously noted after A.C.E. and insulin. The blood sugar levels at 6 hours were higher than at 3 hours in both series, significantly so in the A.C.E. treated rats (Table 2).

It was of interest to determine whether the accentuated urea accumulation after A.C.E. and insulin would occur if A.C.E. treat-

TABLE 3. INFLUENCE OF GLUCOSE ON THE UREA PRODUCTION OF NEPHRECTOMIZED RATS AFTER A.C.E. AND INSULIN

Procedure	No. of rats	*Urea N—Mg N per 100 Gm. B. W./Hour* 0–3 hours	3–6 hours	Change	P
Insulin (0.5 unit per 100 gms) †+ Glucose (500 mg. per 100 gms) † A.C.E. (1.0 ml per 100 gms) *+	8	2.0±0.26	1.8±0.27	−0.2±0.41	>0.5
Insulin (0.5 unit per 100 gms) †+ Glucose (500 mg per 100 gms) †	7	2.4±0.21	2.5±0.20	+0.1±0.37	>0.5

* 0.5 ml A.C.E/100 gms injected at 0 and 1 hours.
† Injected at 3rd hour.

ment were delayed until insulin was given. Table 4 illustrates the results of an experiment in which A.C.E. and insulin were both injected at the 3rd hour. It demonstrates that in the presence of insulin hypoglycemia A.C.E. injection is followed at once by a marked increase in protein catabolism which is sustained during the subsequent 6 hours. This is in contrast to the lack of response of the fasted rat to the same dose of A.C.E. during the first 3 hours after its administration and to the similar delay noted after insulin alone

TABLE 4. INFLUENCE OF SIMULTANEOUS A.C.E. AND INSULIN INJECTION ON THE UREA PRODUCTION OF NEPHRECTOMIZED RATS

Procedure	No. of rats	*Urea N—Mg N per 100 Gm. B. W./Hour* 0–3 hours	3–6 hours	Change	P	6–9 hours	Change	P
A.C.E. (1.0 ml per 100 gms) †	6	1.4±0.26	1.5±0.21	+0.1±0.35	>0.5	—	—	—
A.C.E. (1.0 ml per 100 gms) †+Insulin (0.08 unit per 100 gms) †	8	2.2±0.23	4.8±0.37	+2.6±0.44	<0.01	5.2±0.50	+3.0±0.53	<0.01

† Injected at 3rd Hour.

(Table 1), and believed to be indicative of endogenous adrenal secretion. In the presence of the proper stimulus, i.e., carbohydrate depletion, an increased level of adrenal hormone is associated with a prompt change in protein metabolism. Under different conditions, i.e., fasting or glucose feeding, this same dose of hormone elicits either a small, delayed increase, or no change in protein catabolism.

DISCUSSION

The remarkable increase in urea formation in the nephrectomized rat following treatment with adrenal cortical extract prior to or during insulin hypoglycemia would seem to support the view previously expressed (Engel, Schiller, and Pentz, 1949, Bondy, Engel and Farrar, 1949) that relative depletion of carbohydrate stores and subsequent increased need for gluconeogenesis during fasting is at least one factor which determines the occurrence and magnitude of

the protein catabolic response to A.C.E. This interpretation was based on the finding that the increased urea formation noted after A.C.E. in the fasted nephrectomized rat persisted after the intravenous injection of a fat emulsion or of human serum albumin, but not if glucose or amino acids were injected. During either fasting or following fat injection tissue proteins were the only possible sources of new carbohydrate after A.C.E. treatment. Albumin injected intravenously presumably was indistinguishable from tissue protein as a new source of carbohydrate and hence an increase in urea formation occurred in the rat treated with A.C.E. but not in the untreated animal. After glucose no increase in urea formation occurred in the fasted or albumin injected rat pretreated with A.C.E. No greater amount of urea was formed after the injection of amino acids and A.C.E. than after either one alone and evidence was presented to indicate that the amino acid injection suppressed the tissue protein breakdown customarily seen after A.C.E. All data indicated that the effects of A.C.E., however mediated, were on protein breakdown itself and not on deamination of amino acids, an interpretation supported by the findings in adrenalectomized rats (Bondy, Engel, and Farrar 1949). In order to permit survival after combined adrenalectomy and nephrectomy, a low potassium diet was necessary. Adrenalectomized-nephrectomized rats showed the same rate of urea formation after amino acid injection as did rats with intact adrenals. Under the dietary conditions used here, the nephrectomized rat exhibited an increase in urea formation after the injection of whole protein (rat plasma), a response not obtained in rats on an adequate diet, as noted above. This was attributed, in part, to a disability in storing protein during potassium deficiency. In contrast, the adrenalectomized rat showed no change in urea formation after intravenous protein injection unless A.C.E. were given, indicating that the latter was necessary for the protein catabolism that occurred.

Insulin hypoglycemia in the fasted animal throws a severe strain on the gluconeogenetic mechanisms and hence it was anticipated that if the protein catabolic response to A.C.E. were conditioned by the needs for gluconeogenesis and the non-availability of carbohydrate precursors other than tissue protein, a large increase in urea formation would occur after A.C.E. and insulin in the nephrectomized rat. Such an increase did occur and in contrast to the effect of A.C.E. in the fasted animal, there was no latent period between the time of injection of A.C.E. and the first detectable increase in urea. Either the relative need for gluconeogenesis determines both the speed and the magnitude of the protein catabolic response to A.C.E. or the magnitude of the response to A.C.E. after fasting is so much smaller during the first three hours as to be undetectable by the present methods. The delayed occurrence of an increased rate of urea formation in the rats treated with insulin alone is of considerable interest and suggests that approximately 3 hours must elapse before endog-

enous adrenal cortical hormone secretion has reached levels sufficient to trip off the protein catabolic response. It is well known that insulin hypoglycemia in the fasted animal will increase protein catabolism and stimulate the adrenal cortex (See Tepperman, Engel, and Long, 1943 for review of literature). The present method of study has made it possible to determine the time relations of this response.

As might be anticipated from earlier work, prevention of hypoglycemia by administering glucose simultaneously with the insulin abolished the accelerated protein catabolism after A.C.E. and insulin, as well as that to be expected from A.C.E. alone. The change in urea

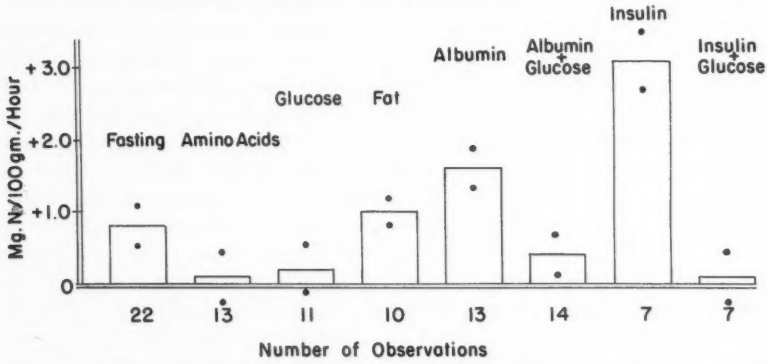

FIG. 1. Mean increases (±S.E.) in urea formation after treatment with 1.0 cc. of A.C.E. The vertical bars represent the differences in urea formation between animals treated with A.C.E. and controls similarly treated but for A.C.E. The data for the first six bars were taken from a previous report (Engel, Schiller and Pentz, 1949).

formation, therefore, cannot be attributed to the insulin per se. In point of fact, insulin in the presence of sufficient glucose to prevent hypoglycemia has an inhibitory effect on protein catabolism. Frame and Russell (1946) and Ingle, Prestrud and Nezamis (1947) showed that insulin will depress the rate of accumulation of amino acids in the blood of eviscerated rats. The evidence all favors the view that the increase in urea formation in the nephrectomized rat after A.C.E., with or without insulin, reflects an increase in protein catabolism that is mediated directly or indirectly by the cortical hormones. It is of interest in this regard that Bondy (1949) has recently shown that A.C.E. will increase the rate of amino acid accumulation in the eviscerated rat and Ingle, Prestrud and Nezamis (1948) report that adrenalectomy in the eviscerated rat lowered the rate of accumulation of amino acids. It is important to know whether glucose will influence amino acid levels in eviscerated rats after A.C.E. treatment.

The observations reported here and in the previous paper (Engel, Schiller and Pentz 1949) throw additional light on the mechanisms involved in the changes in protein metabolism after treatment with

adrenal cortical extract. Figure 1 records the mean increases (±S.E.) in urea formation after the injection of 1.0 cc. of A.C.E. into animals differently treated, and emphasizes the most significant feature of these observations.That is that the injection of the same dose of A.C.E. may be followed by widely different responses depending on the internal metabolic environment of the organism at the time of treatment. Thus, there may be no detectable change in protein catabolism, as reflected in urea formation, in the A.C.E. treated animal given amino acids, glucose, albumin plus glucose or insulin plus glucose, a moderate increase in the fasted, the fat or albumin treated animal and a marked increase during insulin hypoglycemia. These facts suggest that the adrenal cortex may not be the prime mover in changing the rate of protein catabolism. Rather it would appear that a definite level of cortical hormone may be necessary but not responsible for increasing the rate of protein catabolism in response to the proper stimulus but that the magnitude of the response is largely independent of the amount of cortical hormone beyond the stimulating level provided overdosage effects are not involved. This implies that the effects of massive doses of cortical hormone may not be the same as those after physiologic doses. There is ample precedent for such a view. Thyroid is growth promoting in physiological doses, growth inhibiting in excess. Similarly, insulin has different effects at different dose levels. The concept that the adrenal cortex is necessary but not responsible for a change in protein metabolism is the same as that expressed by Ingle, Ward and Kuizenga (1947) with reference to the relation of the adrenal cortex to the catabolic response to injury. They showed that the increase in nitrogen excretion which occurs in normal but not in adrenalectomized animals after a fracture, did occur in the latter if given a constant maintenance dose of A.C.E. Indeed, one may well wonder whether stress in its broadest sense, which would include fasting as well as insulin hypoglycemia in our experiments, may not be one common denominator determining whether an increase in protein catabolism occurs in the presence of cortical hormone. The magnitude of the stress and the availability of new carbohydrate from precursors other than tissue protein might largely determine the magnitude of the protein catabolic response. Since all types of stress are followed by evidence of increased protein catabolism and activation of the adrenal cortex in normal but not in adrenalectomized or hypophysectomized animals (Tepperman, Engel and Long, 1943), whereas stress regularly results in hypoglycemia and death in the latter, it may be that an increased need for carbohydrate is the first effect of stress in general. In the presence of a normally responsive adrenal cortex the counteraction of increased protein catabolism, gluconeogenesis, and decreased carbohydrate utilization would then take place as existing stores of carbohydrate begin to become depleted. It should be emphasized that these interpretations of the role of the adrenal cortex in metabolism

refer to protein metabolism alone. In this regard, however, it is of interest that Levin (1949) has recently reported that the increase in liver fat, which begins within an hour of physical stress or injection of adrenocorticotrophic hormone, and which he believes is mediated through the pituitary-adrenal system, is prevented by parenteral administration of glucose during the stress or after A.C.T.H.

Further investigation is in progress with respect to the role of stress in the protein catabolic response to A.C.E.

SUMMARY

The effects were studied of insulin and adrenal cortical extract (A.C.E.) on protein catabolism, measured as the rate of urea accumulation in nephrectomized rats.

Both A.C.E. and hypoglycemic doses of insulin each caused a delayed increase in urea formation beginning 3 hours after injection. The response to insulin was significantly greater than that to A.C.E. and was interpreted as being related to endogenous cortin production.

Either pretreatment with A.C.E. followed in 3 hours by insulin or simultaneous injection of A.C.E. and insulin resulted in a marked increase in urea formation beginning immediately after injection of insulin. The increase after A.C.E. plus insulin was 3–4 times as great as after A.C.E. alone, but not significantly greater than the delayed response to insulin.

Glucose prevented both the hypoglycemia and the increase in urea formation seen after insulin and A.C.E.

Since hypoglycemia greatly accentuates the needs for new carbohydrate, the finding of greatly accelerated protein catabolism after insulin and A.C.E., compared to A.C.E. alone, supports the view that the need for new carbohydrate is one factor which determines the occurrence and magnitude of the protein catabolic response to A.C.E. The possible role of nonspecific stress in this response is considered and the possibility suggested that this may be the factor which determines the protein catabolic response for which the adrenal cortex is necessary but not itself responsible.

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TROPHIC HORMONES OF THE PLACENTA: FAILURE TO DEMONSTRATE THYROTROPHIN OR ADRENOCORTICOTROPHIN PRODUCTION IN THE HYPOPHYSECTOMIZED PREGNANT RAT[1]

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IT HAS BEEN KNOWN since the work of such investigators as Allan and Wiles (1932) using the cat and Pencharz and Long (1933) using the rat that pregnancy can continue in gravid animals after hypophysectomy. This observation has been confirmed in many different species such as guinea pigs, mice, dogs, rabbits, and monkeys. It is now well established, of course, that pregnancy is able to continue because the placenta takes over in part the functions of the pituitary gland and ovary. In the lower mammals investigated, the placental hormone chiefly responsible for maintaining pregnancy seems to be a luteotrophin, since ovariectomy in animals such as the rat results in abortion or resorption of the foetuses in hypophysectomized pregnant animals although pregnancy continues undisturbed if the ovaries are left intact. In certain species including the primates, the placenta is able also to take over the function of the ovaries, since ovariectomy will not terminate pregnancy and estrogen and progesterone have been isolated from placental tissue of the higher mammals.

It is a curious fact that the pregnant animals deprived of their pituitaries seem to do remarkably well in comparison to the notably poor behavior of the hypophysectomized non-pregnant animals. For instance, Gardner and Allen (1942) found that mice hypophysectomized on the tenth day after mating maintained parallel increases in body weight with their non-operated pregnant controls. It is almost as if the pregnant animal were able to maintain a normal endocrine balance in the absence of the pituitary. Except for the discoveries that the placenta secretes a luteotrophin (Astwood and Greep, 1938), estrogen (Doisy *et al.*, 1924), progesterone (Ehrhardt, 1934), and androgen (Cunningham and Kuhn, 1941), little work has been done on the problem of other endocrine functions of the placenta.

Because the pregnant rat seems to thrive in the absence of the pituitary and since the rat placenta is apparently unable to substitute

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for the ovaries by secreting sufficient amounts of estrogen and progesterone to maintain pregnancy, the present investigation was undertaken to determine whether the pregnant rat is able to maintain endocrine glands other than the corpus luteum in a normal functional state. Since quite satisfactory methods are now available for evaluating thyroid and adrenal function, this work was limited to a study of whether or not a thyrotrophin or an adrenocorticotrophin are secreted in the hypophysectomized pregnant rat.

TABLE 1. THYROID/SERUM (T/S) IODIDE RATIO AND THE WEIGHTS OF CERTAIN ENDOCRINE GLANDS IN MG./100 GM. BODY WEIGHT

	Number of animals	T/S	Adrenal	Ovary	Thymus	Thyroid
I Pregnant, intact	9	219.6±29.9	22.8±1.4	40.2±1.6	59.9± 5.5	14.1±1.1
II Pregnant, hypophysectomized	9	6.4± 0.7	18.6±0.6	37.8±1.8	95.3±16.3	6.6±0.5
III Non-pregnant, hypophysectomized	10	6.1± 1.1	20.4±1.6	21.6±0.8	139.3±13.7	7.0±0.6

MATERIALS AND METHODS

Female, 150–200 gm., Sprague-Dawley rats of the Harvard strain were used in all experiments. Thirty-five rats were used in all, but some had to be discarded because of incomplete hypophysectomies. The rats were divided into three groups: Group I consisted of nine unoperated pregnant rats, Group II contained nine pregnant rats which were hypophysectomized by the parapharyngeal approach on the twelfth to thirteenth day after mating, Group III comprised ten non-pregnant hypophysectomized animals. All pregnant rats were killed by ether anesthesia on the twenty-first day of pregnancy. The non-pregnant animals were killed in the same manner on the eighth to ninth day following hypophysectomy. The hypophysectomized animals were given 0.03 per cent propylthiouracil mixed in mink chow immediately following the operation. The intact, pregnant animals were placed on the propylthiouracil diet on the twelfth to thirteenth day of pregnancy. Prior to the administration of the antithyroid drug the animals were fed on Purina fox checkers. Body weights of the pregnant animals were determined after removal of the foetuses and placentae. The thyroids, adrenals, ovaries, and thymus were removed, dissected free of fat and connective tissue on moist blotting paper and immediately weighed to the nearest 0.1 mg. on a Roller-Smith torsion balance, following which all except the left lobe of the thyroid were deposited in 10 per cent formalin.

The thyroid/serum iodide ratio was determined in the following manner. Each animal was given a subcutaneous injection of 5 mg. propylthiouracil in an alkaline, aqueous solution on the last day of the experiment. Thirty to sixty minutes later a tracer dose of 2 to 3 microcuries of carrier-free I^{131} was injected subcutaneously. One hour later the animals were killed with ether anesthesia as described above. Heart blood was obtained, either by cardiac puncture or by opening the thorax and incising the heart directly, and this was centrifuged to obtain the serum. 0.1 cc. of this serum was pipetted onto

a piece of absorbent paper not quite covering a one-inch standard copper disc. The serum was evaporated to dryness under an infra-red lamp and the paper then secured in place by covering with a single layer of Scotch Tape.

The left lobe of the thyroid gland was placed on a similar piece of absorbent paper on a copper disc immediately after weighing. It was then covered with a single layer of Scotch Tape and the gland smashed flat by pressing with a heavy weight. The radioactivity of each sample was determined with a mica-windowed, Geiger-Müller counter. The radioactivity per 100 mg. of each sample was then computed, and the thyroid/serum (T/S) iodide ratio thereby determined.

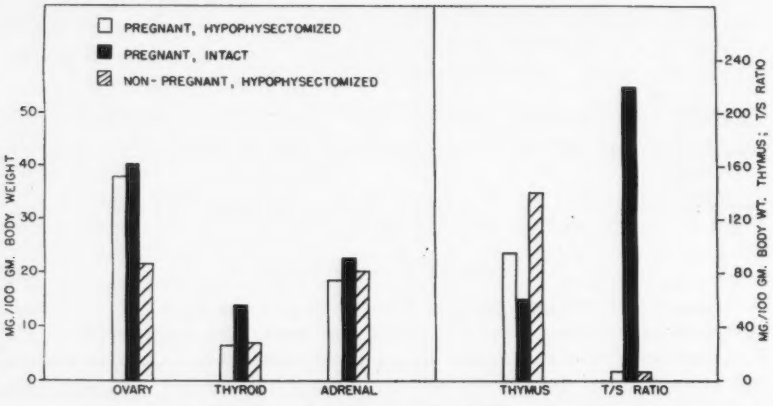

FIG. 1. Graphic comparison of certain organ weights and the thyroid/serum iodide ratio of the three groups of experimental animals.

After fixation in formalin for at least one week, the adrenal glands were washed in running water for an hour and sectioned on the freezing microtome at 15 micra.[2] One of the sections was stained with sudan IV, one with sudan black B, and a third treated with the Schiff plasmal reagent. An unstained section and a control section extracted for one-half hour in cold acetone were examined in the polarizing and fluorescent microscopes to determine the quantity and distribution of acetone-soluble, birefringent, and auto-fluorescent materials in the cortex. The lipids which react in a positive manner to all of these tests are generally conceded to give a fair indication of the hormone present in the gland.

After the animals had been killed, the cranium was opened and the region of the sella turcica examined carefully under a dissecting microscope in order to determine the completeness of hypophysectomy. All operated animals in which there was any question of a pituitary fragment remaining were excluded.

RESULTS

As had been found by previous investigators, the hypophysectomized pregnant animals experienced no difficulty in carrying their

[2] The adrenal sections were kindly prepared through the courtesy of Dr. R. O. Greep.

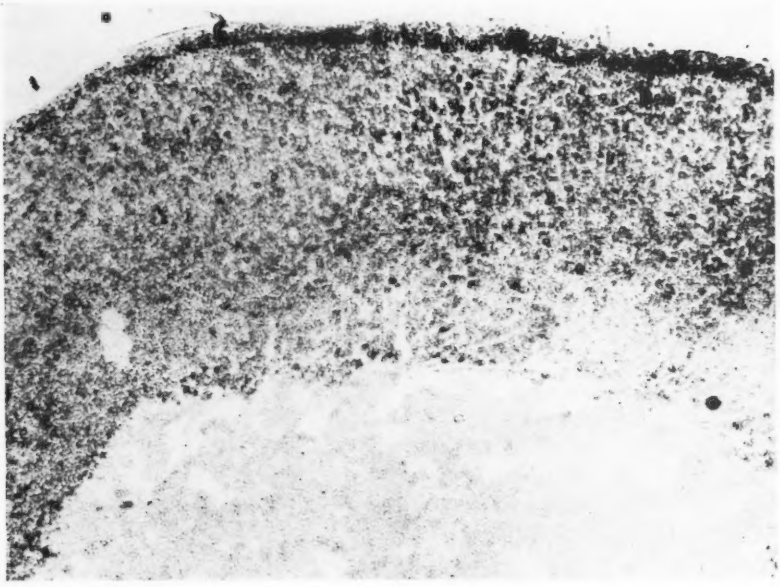

FIG. 2. Sudan black B stain of frozen section of adrenal from intact pregnant rat. The glomerulosa and fasciculata are directly contiguous without any intervening unstained cells.

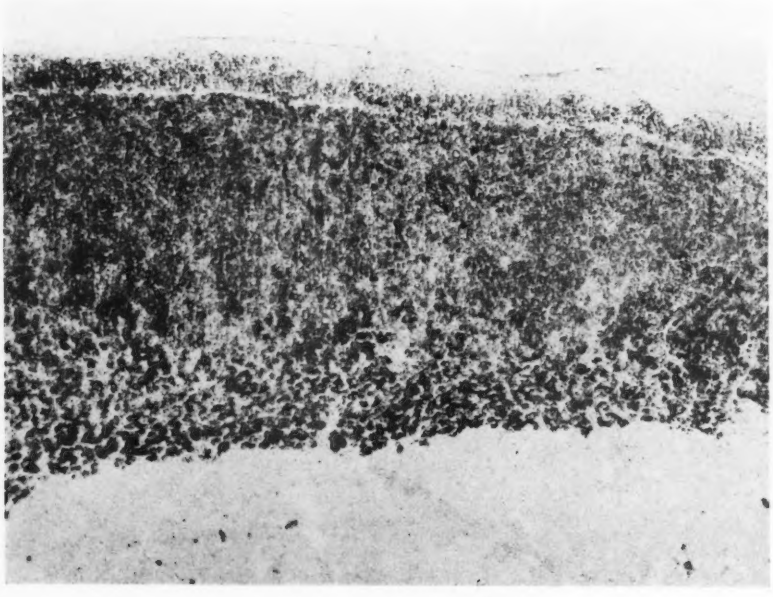

FIG. 3. Sudan black B stain of adrenal from hypophysectomized pregnant rat. Note the appearance of a "lipophobic" zone between the glomerulosa and fasciculata.

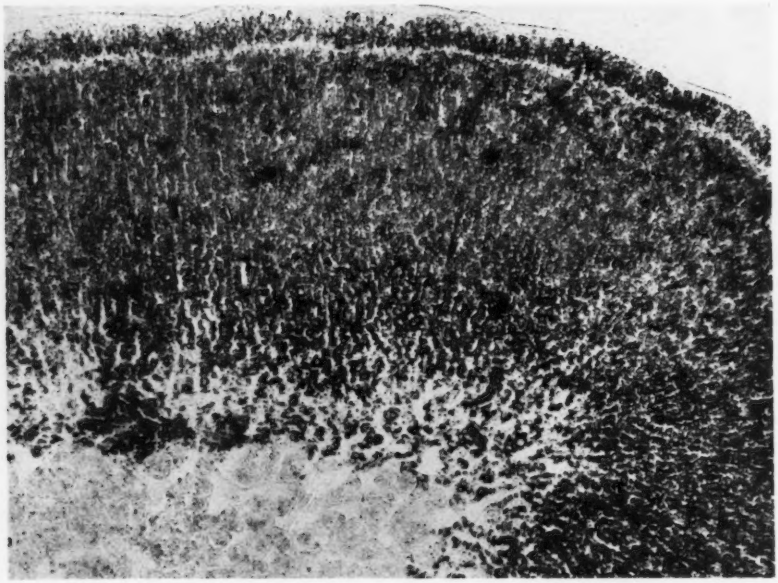

FIG. 4. Sudan black B stain of adrenal from hypophysectomized non-pregnant rat. The appearance is identical to that in Figure 3.

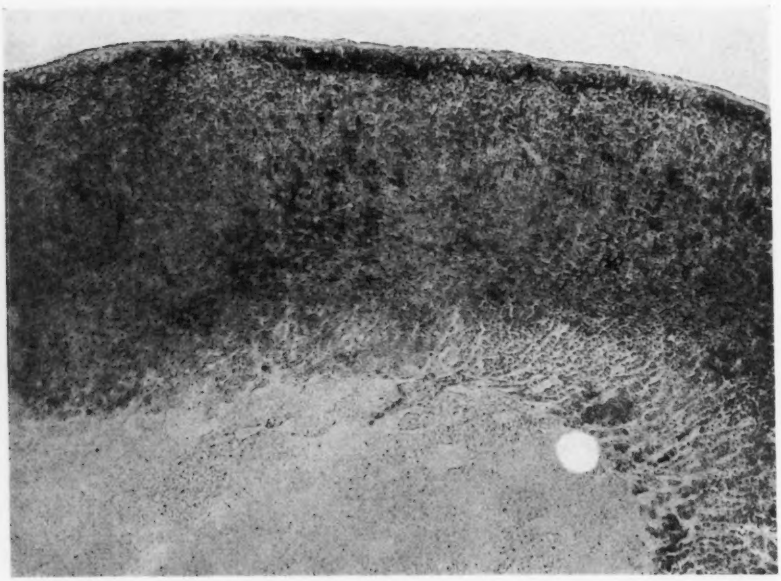

FIG. 5. Schiff stain of adrenal from intact pregnant rat. Note that the glomerulosa is more intensely stained than the fasciculata.

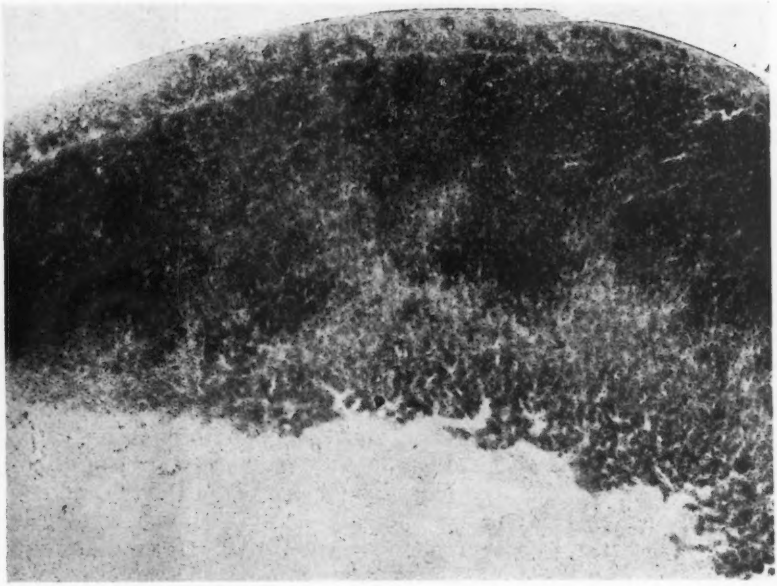

FIG. 6. Schiff stain of adrenal from hypophysectomized pregnant rat. In contrast to Figure 5, the fasciculata is much more intensely stained than the glomerulosa.

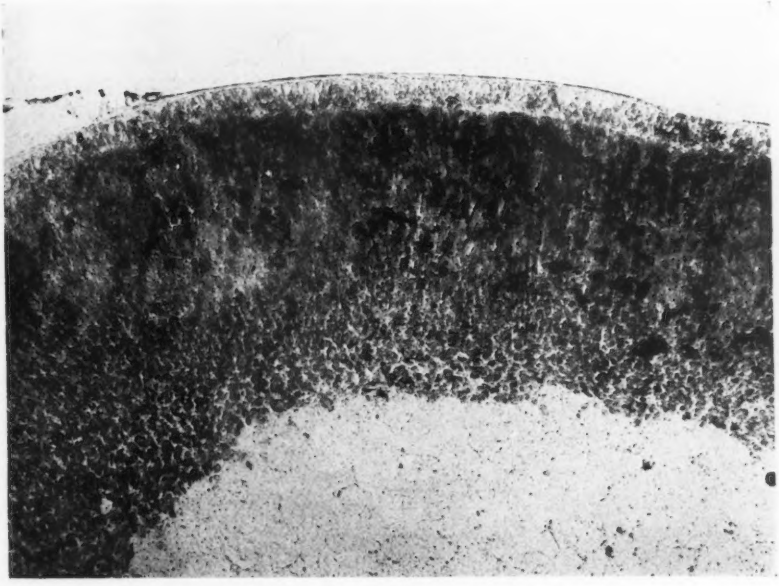

FIG. 7. Schiff stain of adrenal from hypophysectomized non-pregnant rat. Appearance is identical to that of the adrenal from the hypophysectomized pregnant rat illustrated in Figure 6.

foetuses to term. Since Pencharz and Long (1933) had shown that hypophysectomy prior to the eleventh day of pregnancy usually resulted in resorption or expulsion of the foetuses, the twelfth to thirteenth day after mating was chosen for the operation in these experiments. In no instance was any resorption or maceration of any of the young noted at autopsy, nor was the number of animals per litter reduced below the figure for the controls. The body weights of the hypophysectomized pregnant animals were comparable to those of the intact animals, while the hypophysectomized non-pregnant animals seemed to be somewhat more emaciated.

Thyrotrophin

VanderLaan and VanderLaan (1947) have shown that the normal thyroid/serum iodide ratio of 25:1 could be increased ten-fold by placing rats on a diet of 0.03 per cent propylthiouracil for ten days or longer. Recently, VanderLaan and Greer (to be published) have shown that this marked increase in the iodide-concentrating power of the thyroid was due to the increased efficiency of the thyroid cells when stimulated by the large amounts of thyrotrophin secreted by the pituitary of a hypothyroid rat. Hypophysectomy would reduce the T/S ratio to the normal level within two days in rats continued on propylthiouracil. The iodide-concentrating mechanism was independent of the cellularity of the gland and of its relative mass. Thyrotrophin injection would cause an increase in the T/S ratio similar to that produced by chronic propylthiouracil administration.

The determination of the thyroid/serum iodide ratio therefore seemed an excellent method for estimating just how much thyrotrophin was being produced in these animals. It was reasoned that the feeding of propylthiouracil from the time of hypophysectomy until the time of autopsy would serve to accentuate any differences between the groups which might exist. If, as seemed unlikely, the placenta were capable of secreting as much thyrotrophin in response to the stimulus of thyroxin-lack as was the pituitary, the hypophysectomized pregnant animals would exhibit T/S ratios comparable to the markedly elevated levels in the intact hypothyroid animals. On the other hand, if the placenta were able to secrete only enough thyrotrophin to maintain a euthyroid state under normal conditions a T/S ratio of around 25:1, approximating that of untreated intact animals, would be expected. If no thyrotrophin were secreted by a pregnant rat in the absence of the pituitary, the T/S ratio should fall well below 25:1.

a) *Pregnant, intact animals:* The increase in the T/S ratio observed in these animals was no different from that previously observed in non-pregnant, thiouracil-treated rats. The mean value was 219.6:1 (range 89.3–382).

b) *Pregnant, hypophysectomized animals:* These animals gave no

indication of any extra-pituitary source of thyrotrophin. The mean ratio was 6.4:1 (range 3.3–13.6) which represents a level only one-fourth that of intact animals which were not treated with thiouracil.

c) *Non-pregnant, hypophysectomized animals:* As expected, no evidence of thyrotrophin production in these animals was found. The mean T/S level was 6.1:1 (range 1.8–10.1) almost identical to that in b), above.

Adrenocorticotrophin

Unfortunately, no reliable and simple method has yet been found for directly measuring the adrenocorticotrophin production in small animals. Histochemical methods, however, are generally conceded to give a fairly reliable index of adrenal cortex activity. Adrenal weights have been considered to be of great value in estimating the amount of adrenocorticotrophin present, but in these experiments there was very little difference between the weights of the adrenals in any of the three groups. This may well have been because all animals were receiving antithyroid medication for it has been well established (Deane and Greep, 1947) that thiouracil will cause a decrease in adrenal size.

In any event, examination of the adrenal sections showed that although the intact pregnant rats had perfectly normal appearing glands, both the pregnant and non-pregnant hypophysectomized animals showed changes in the cortex characteristic of rats operated on in this manner. There was a narrowing of the fasciculata, and the lipoid droplets in this region were much more diffuse and less localized in intensely staining cells than in the intact animals. A characteristic lipophobic zone appeared between the glomerulosa and fasciculata of the hypophysectomized animals which was not seen in the intact animals. The Schiff reagent stain revealed a darker staining glomerulosa and lighter staining fasciculata for the intact animals and just the reverse for both groups of operated animals. It was concluded, therefore, that no adrenocorticotrophin could be detected in the hypophysectomized pregnant rat.

Weights of endocrine glands

Although they were not used as such in this investigation, the weights of organs which depend upon the pituitary for their function have frequently been employed as an index of pituitary activity. The findings in these experiments also show that in the absence of trophic hormones the "end-organs" tend to atrophy. For instance, the thyroid and adrenal weights in the hypophysectomized animals are below those of the intact rats, although in the case of the adrenals it is questionable whether a significant difference exists. The thymus, which has been observed to enlarge after adrenalectomy or hypophysectomy, was also a good deal larger in the hypophysectomized than

the intact rats in these experiments. The ovaries, on the other hand, which were stimulated by the placental gonadotrophins, were markedly larger in both groups of pregnant rats than in the non-pregnant. The large size of the thyroids of the intact pregnant animals, of course, was due to the administration of propylthiouracil.

DISCUSSION

These results strongly indicate that neither thyrotrophin nor adrenocorticotrophin is produced in the hypophysectomized pregnant rat. In the only other investigation along a similar line which could be found in the literature, Gardner and Allen (1942) observed that the adrenals of pregnant mice which were hypophysectomized on the tenth day after mating and killed on the first to second day after parturition had adrenals weighing only 3.8 mg. compared to 6.9 mg. for the pregnant, intact controls. The loss of adrenal substance in their experiment was also chiefly in the fasciculata. The fact that they observed more adrenal atrophy in their work than occurred in the present investigation may have been due to any or all of three factors: 1) a species difference may exist between the rate of atrophy of the adrenals of mice and rats; 2) their animals were allowed to go two to four days longer after hypophysectomy than those in the present experiment; 3) our rats received propylthiouracil beginning at the time of operation which may have caused a reduction in adrenal size of the intact animals that would lessen any apparent loss of substance due to hypophysectomy (Deane and Greep, 1947).

If adrenocorticotrophin and thyrotrophin are not secreted by the placenta, the difficulty still arises in trying to explain why these pregnant hypophysectomized animals are able to maintain themselves so well. It is possible that although trophic hormones other than gonadotrophins are not secreted by the placenta, other hormones, such as adrenal steroids, etc., might be and would thus explain the maintenance of parallel weight gains of the hypophysectomized with the intact pregnant animals. Since steroids such as progesterone, estrogen, and androgen have been found in placental tissue, it does not seem too far fetched to postulate that other steroids as well as certain non-steroid hormones similar to gonadotrophins may yet be discovered in this organ. That the good health of the hypophysectomized pregnant rats may have something to do with the placenta other than its luteotrophic function is further indicated by the work of Cutuly (1942) who found that in hypophysectomized rats in which pregnancy was maintained by injections of pituitary synergist or lactogenic hormone the animals gained in weight as did their unoperated, pregnant controls. After delivery or after the foetuses had died in utero, however, there was a precipitate decrease in body weight.

SUMMARY

Pregnant rats were hypophysectomized on the twelfth to thirteenth day after mating and given a 0.03 per cent propylthiouracil diet. They were killed on the twenty-first day of pregnancy. The level of thyroid function was determined by the thyroid/serum iodide ratio and of adrenal function by histochemical studies. No evidence of thyrotrophic or adrenocorticotrophic activity in the animals thus treated was found.

ACKNOWLEDGMENTS

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THE EXCRETION OF PANTOTHENIC ACID AND ASCORBIC ACID BY INTACT AND ADRENALECTOMIZED RATS ON DIETS SUPPLEMENTED WITH AND DEFICIENT IN PANTOTHENIC ACID

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INTRODUCTION

PROLONGED survival of adrenalectomized rats receiving 1% NaCl as drinking water and a diet supplemented with at least 4 mg. of calcium pantothenate daily has previously been reported (Ralli, 1946; Dumm and Ralli, 1948). These rats appeared well, gained weight, and were capable of muscular work. In view of the findings, studies are being conducted in adrenalectomized rats to establish the role that pantothenic acid plays under these circumstances. The present report is concerned with the excretion of pantothenate and of ascorbic acid by adrenalectomized and intact rats on diets deficient in and supplemented with calcium pantothenate.

EXPERIMENTAL

All rats were bred in the laboratory. To deplete the rats of pantothenic acid they were placed on a diet deficient in pantothenic acid (Ralli and Graef, 1943; Dumm and Ralli, 1948) at 30 days of age and were continued on this diet for 30 days. The excretion of pantothenate was determined at intervals during this period. At 60 days of age a group of the animals were adrenalectomized and these, as well as the remaining intact rats, were then placed on a diet supplemented with 4 mg. of pantothenic acid daily and 1% NaCl as drinking water. The excretion of pantothenate was determined in both groups of rats at intervals for 115 days. Following this period 4 adrenalectomized and 2 intact rats were returned to the pantothenic acid deficient diet and the excretion of pantothenate was followed for another 30 days.

The excretion of ascorbic acid was determined in all groups of rats under the same conditions of diet and adrenalectomy. In addition ascorbic acid excretion was determined in a group of normal male and female rats at different ages while on the Nuchow diet. This was done in order to establish the effect of sex and age on ascorbic acid excretion.

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All 24-hour urines were collected from single rats in metabolism cages. Specimens to be assayed for pantothenate were preserved with toluene. Urines in which ascorbic acid was to be determined were collected with 1 cc. of 5 N H_2SO_4 (containing .01 M hydroxyquinoline) for each 10 cc. of urine expected.

The pantothenate determinations were carred out by microbiological assay methods (Atkin *et al.*, 1944; Skeggs and Wright, 1944) in the Nutrition Laboratories of Hoffmann-LaRoche, Inc., under the direction of Dr. Saul H. Rubin. Ascorbic acid was determined in the urine by the method described by Rowe and Kuether (1943).

TABLE 1. THE EXCRETION OF PANTOTHENIC ACID BY ADRENALECTOMIZED AND INTACT RATS

Pantothenic Acid Intake 4.0 Mg./Day Following a 30-day Deficient Period

Intact Rats				Adrenalectomized Rats		
Age days	Days on Pan. ac.	Pan. ac. excreted mg.	% Pan. acid excreted	Days after adr. & on pan. acid	Pan. acid excreted mg.	% Pan. acid excreted
63	5	2.5	61	5	3.3	82
64	6	3.0	75	6	4.0	100
65	7	3.5	89	7	1.6	41
66	8	2.4	59	8	2.9	73
72	14	1.8	46	14	1.9	48
72	14	2.0	48	14	2.8	71
79	21	1.6	41	21	1.9	48
79	21	2.3	57	21	2.4	60
175*	115	1.9	41	115	2.3	62
Mean ± S.D.			60 ± 15			65 ± 20

Excretion of pantothenic acid after 20–30 days on the deficient diet was 0.002 to 0.005 mg. per rat per day.

* Rats had been receiving 3 mg. pantothenic acid daily at this time.

RESULTS

Pantothenate Excretion

After 20 to 30 days on the diet deficient in pantothenic acid the excretion of pantothenate varied from 0.002 to 0.005 mg. per day per rat. The data on the excretion of pantothenate by the intact and the adrenalectomized rats receiving 4 mg. of pantothenic acid daily are summarized in Table 1. Both groups of rats excreted about 60% of the pantothenate intake and there was no significant difference in excretion between the intact and adrenalectomized rats. The excretion of pantothenate in the adrenalectomized and intact rats following withdrawal of pantothenic acid from the rat at 119 days is shown in Figure 1. The logarithm of the pantothenate excreted in mcgm. per day is plotted against the time in days during which the diet was deficient in pantothenate. Ten days after stopping the vitamin the excretion had decreased to 0.005 to 0.013 mg. daily. It is clear that during this second period of deficiency the excretion of pantothenate by

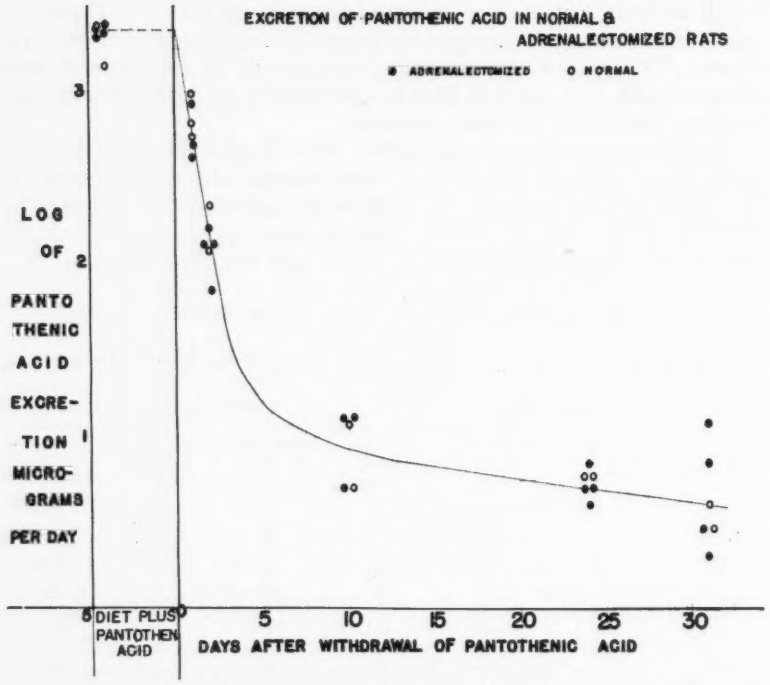

FIG. 1. Excretion of pantothenate by normal and adrenalectomized rats. The logarithm of the pantothenate excreted in micrograms per 24 hours is plotted against the time in days during which the diet was deficient in pantothenate. The rats had been maintained on a diet supplemented with 3 mg. of pantothenate daily for 119 days before pantothenate was withdrawn from the diet.

both the intact and adrenalectomized rats was essentially the same. At the conclusion of this experiment the completeness of adrenalectomy was checked by withdrawing NaCl. All adrenalectomized rats died within 3 days of NaCl withdrawal.

Ascorbic Acid Excretion

In Figure 2 data are presented on the excretion of ascorbic acid by normal rats from 30 to 103 days of age on the Nuchow diet. There were 12 rats in the group studied, 6 males and 6 females. Each point represents an average of 3 to 12 determinations. The ascorbic acid excreted is expressed as mg. per 24 hours per 100 gm. of body weight of the rat and is plotted against the age of the rat in days. All values were corrected for the small amount (7 mcgm/gm.) of ascorbic acid present in the Nuchow diet.

The excretion of ascorbic acid at 30 days of age was about 2 mg. daily and this increased to a maximum of 3.01 ± 1.40 for males and 4.08 ± 0.60 for females at about 45 days of age. Thereafter the ascorbic

acid output decreased rapidly for about 20 days (1.86 ±0.84 for males; 2.15 ±0.58 for females at 63 days) and then continued to fall more slowly for the duration of the study. The excretion of ascorbic acid between 40 and 50 days of age was greater by the female than by the male rats but the difference between the sexes may not be significant ($t = 1.875$; $p = <0.1, >0.05$). After 65 days the average output of ascorbic acid by the male and female rats was approximately the same (1.40 ±0.57 for males; 1.30 ±0.93 for females at 92 days) when expressed with reference to the body weight of the aninals.

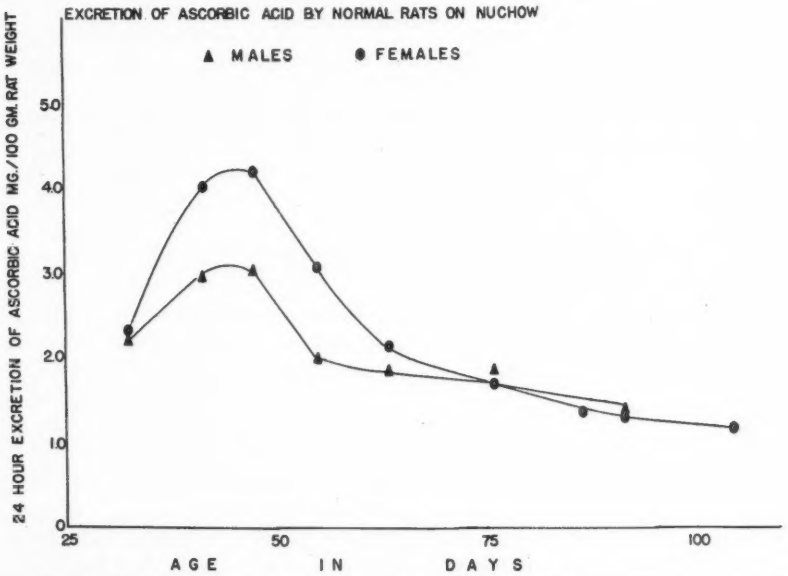

FIG. 2. Excretion of ascorbic acid by normal rats on Nuchow. The excretion of ascorbic acid in milligrams per 100 grams of rat weight per 24 hours is plotted against the age of the rat in days.

The excretion of ascorbic acid by the adrenalectomized rats and their intact controls on the experimental diet supplemented with calcium pantothenate daily is shown in Figure 3. Each point represents an average of 4 to 14 determinations at the indicated ages. It should be noted that the ordinate units in Figure 3 represent half as much ascorbic acid excreted as the ordinate units in Figure 2. The data indicate that the adrenalectomized rats excreted less ascorbic acid at all ages examined than the intact rats on the same diet. At 170 days, for example, the intact rats excreted 0.69 ±0.25 mg. and the adrenalectomized rats 0.27 ±0.09 mg. of ascorbic acid daily. The intact rats showed a maximum excretion of ascorbic acid between 80 and 90 days of age (1.41 ±0.36 mg.). The adrenalectomized rats also excreted slightly more ascorbic acid between 80 and 90 days of age than during

any other time studied, but the difference between 85 days (0.53 ± 0.12 mg.) and either 75 (0.25 ± 0.10 mg.) or 95 (0.28 ± 0.18 mg.) days is probably not significant ($t = 1.556$; $p = <.2, >.1$).

Table 2 shows the excretion of ascorbic acid during part of the pantothenate withdrawal experiment described in connection with Figure 1. The daily excretion of ascorbic acid was determined for each rat 6 and 13 days before pantothenate was discontinued and on the

TABLE 2. EXCRETION OF ASCORBIC ACID
Mg. per 100 Gm. Body Weight per 24 Hours

Pantothenate per day, mg.	Days after pantothenate discontinued	Adrenalectomized				Intact	
		Rat A-1	Rat A-2	Rat A-3	Rat A-4	Rat I-1	Rat I-2
3	–	0.17	0.17	0.25	0.32	0.56	0.56
3	–	0.17	0.15	0.27	0.37	1.07	0.56
0	4	0.16	0.17	0.23	0.38	0.57	0.55
0	8	0.21	0.18	0.28	0.32	0.99	0.49

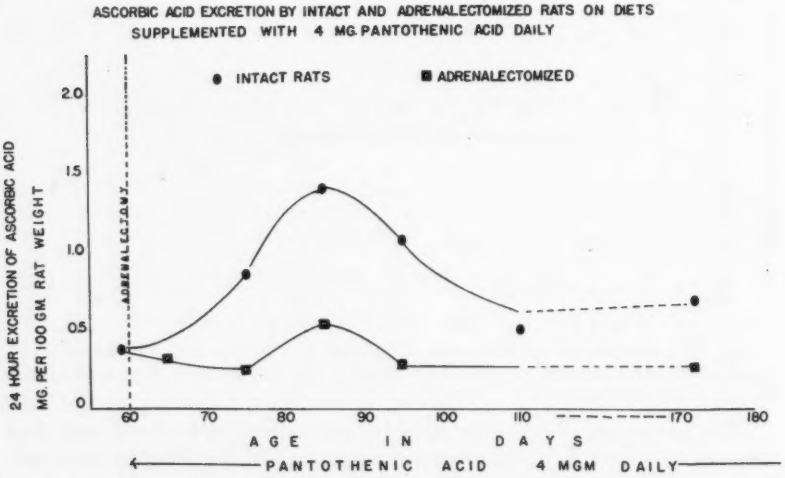

FIG. 3. The excretion of ascorbic acid by intact and adrenalectomized rats on a diet supplemented with 4 mg. pantothenate daily. The excretion of ascorbic acid in milligrams per 100 gms. of rat weight per 24 hours is plotted against the age of the rat in days. The rats received a diet deficient in pantothenate from 30 to 60 days of age and a diet supplemented with 4 mg. pantothenate daily after 60 days of age.

4th and 8th day afterward. All the adrenalectomized rats maintained a constant and low output of ascorbic acid whether or not pantothenic acid was included in the diet. More fluctuation was encountered in the ascorbic acid excretion of the intact rats, but there was no indication of any consistent change following the withdrawal of pantothenic acid in either group of animals.

DISCUSSION

In spite of the high requirement by adrenalectomized rats for pantothenic acid (Ralli, 1946; Dumm and Ralli, 1948) the data presented on its excretion by intact and by adrenalectomized rats showed no differences between the 2 groups, either at high intakes of the vitamin or in the withdrawal experiment. Both groups of rats excreted a far higher proportion of the dose of calcium pantothenate administered than has been reported in human subjects (Rubin *et al.*, 1948; Gershberg, Rubin and Ralli, 1949).

The excretion of ascorbic acid, on the other hand, was influenced both by the nature of the diet and by the removal of the adrenal glands, the latter being associated with the lowest excretion of ascorbic acid encountered. These findings are of interest for several reasons. Ascorbic acid is synthesized by the rat and is not a dietary requirement for this animal. The data presented suggest that the adrenal gland is involved in the synthesis of ascorbic acid. This is demonstrated in both of the experimental conditions to which the rats were subjected. From 30 to 60 days of age the rats received a diet deficient in pantothenic acid, a deficiency which in young rats has been shown to cause lipid depletion of the adrenal cortex (Deane and McKibben, 1946). The 24-hour excretion of ascorbic acid at the end of this period was 0.37 ± 0.18 mg. per 100 gm. of rat weight as compared to 2.00 ± 0.72 mg. by normal rats of the same age on Nuchow. When the deficient rats were adrenalectomized, the ascorbic acid continued at a very low level in spite of the addition of large amounts of pantothenate to the diet, whereas the excretion by the intact controls rose although not to the level encountered in the intact rats on Nuchow. In the intact experimental rats the excretion levelled off at about 110 days and averaged 0.9 mg. per 100 gm. of rat weight. In the adrenalectomized rats, however, the level of excretion, except for the slight increase at 85 days of age, remained at an average of 0.26 mg. per 100 gm. of rat weight and was remarkably constant, as can be seen in Table 2.

Other observers have reported that the composition of the diet can influence the excretion of ascorbic acid and have found that the cyclic compounds of the terpene and sesquiterpene series stimulated a marked increase in the amounts of ascorbic acid excreted by normal rats (Longenecker *et al.*, 1939). In our experiments the dietary variant was pantothenic acid, and although a deficiency of this substance in the young rats was associated with a marked decrease in the ascorbic acid excretion, its withdrawal from the diet of the rats, once they had reached maturity, was not associated with any striking change in excretion. This may reflect the fact that the requirement for pantothenic acid decreases with age in the rat (Unna and Richards, 1942).

There is good evidence that the age of the animals is a factor in the excretion, and probably the synthesis, of ascorbic acid. In the normal

rats the daily excretion of 3–4 mg. ascorbic acid between 40 and 50 days of age was followed by a much lower output (< 2mg.) after 60 days. The greatest excretion of ascorbic acid occurred just prior to the development of sexual maturity, which usually occurs between 50 and 60 days of age (Farris, 1942), suggesting that the activity of other endocrine glands is associated with the utilization and synthesis of vitamin C. This is also borne out by the work of other investigators. Plasma ascorbic acid was increased during oestrus in the cow (Phillips *et al.*, 1941) and was decreased in both the cow and the bull following the injection of gonadotropin (Erb and Andrews, 1942).

The effect of pantothenic acid on survival in adrenalectomized rats is not explained by these experiments. Apparently the decreased synthesis of ascorbic acid, as reflected by the low excretion, does not jeopardize the survival. Experiments on the acetylation of para-aminobenzoic acid are now being done and will be reported later.

SUMMARY

The data presented show that the survival of adrenalectomized rats, which occurs when they receive NaCl and large doses of pantothenic acid, is not associated with any difference in the excretion of pantothenic acid as compared to intact rats on similar diets. The excretion of ascorbic acid, however, is lower by the adrenalectomized rats receiving pantothenic acid than by their controls, but this lower excretion is apparently not a factor detrimental to prolonged survival. There was never any evidence in the animals of signs suggestive of ascorbic acid deficiency.

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THE EFFECT OF FOLLICLE STIMULATING HORMONE UPON OXYGEN CONSUMPTION OF CHICK OVARY SLICES[1]

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THE GONADOTROPHIC FUNCTION of the pituitary gland has been the subject of many investigations by endocrinologists but these studies have for the most part been limited to measurement of anatomical and gross physiological responses of the gonads. There has been little work done to elucidate the mechanism by which the pituitary gonadotrophic hormones produce these effects. It is known that the follicle stimulating hormone (FSH) promotes the growth of ovarian follicles but it is not known how FSH affects cellular processes to bring about this end result. Therefore, it seems of interest to measure responses of ovarian tissue to FSH at the cellular level. Since FSH stimulates the ovary to greater activity measurable as increase in weight and rate of function one might expect an increased rate of respiration of ovarian tissue in response to the hormone. In this study the effect of FSH on the oxygen consumption of chick ovary slices has been measured.

MATERIALS AND METHODS

The rate of oxygen uptake by chick ovary slices was measured by means of the Warburg apparatus. In most of the experiments White Leghorn chicks two to three months of age were used but in a few cases cross-bred chicks of a similar age were used. The animals were killed by luxating the cervical vertebrae. The ovaries were removed quickly, cleaned of the adhering adrenals and covering membranes, and placed in cold Krebs-Ringer solution buffered with phosphate to pH 7.2. The ovaries were sliced with a razor blade as quickly as possible, the lapse of time between killing of animals and taking of zero readings on the manometers being approximately one hour. The flasks containing the tissues were kept cold until they were placed in the water bath. Either four or six chicks were used in each run. The total number of animals used in the study was 351. If six flasks were used in a run ovaries from six chicks were used and the slices from these were distributed in such a way that each flask received a slice from each ovary and the slices in each

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[2] Postdoctoral Fellow of the University of Illinois Graduate School, Oct. 1947–June 1948; National Institutes of Health Postdoctoral Fellow, June 1948–June 1949.

flask were comparable in their position in the ovary (that is, each flask received a top slice, a second slice, a third, etc.). Manometer readings were taken at twenty-minute intervals for one hour. When manometric measurements were completed the tissues were removed from the flasks, dried overnight at 90°C., and weighed. The amount of tissue in each respiration flask was usually around 10 mg. dry weight. QO_2 was calculated as mm.^{3}/mg. dry weight tissue/hour. Suspension media used were Krebs-Ringer solution buffered with phosphate to pH 7.2, and the same solution containing glucose in concentration of 0.02 M. or sodium pyruvate in concentration of 0.018 M. The respiration flasks contained 3 ml. of suspension medium in the main compartment and 0.2 ml. of 5% KOH in the center well. When hormone was added to the system the total dose was usually dissolved in 0.3 ml. of the suspension medium and this placed in the side-arm of the flask, the volume of the medium in the main compartment being reduced to 2.7 ml. The hormone solution was added to the main compartments of the flasks just before they were placed in the water bath. All measurements were made in an atmosphere of pure oxygen and at 40.5°C. which is approximately the body temperature of chickens. The biological activity of the hormones used in this study was determined on 21-day old female rats (Table 1).[3]

TABLE 1. BIOASSAY OF HORMONE PREPARATIONS

Prepn.	Total dose (mg.)	Time injected (days)	Rats		Av. wt. ovaries (mg.)	Response ovary
			Kind	No.		
FSH 5367	7.8	4.5	Normal	12	45	Follicles only
FSH 534	5.7	4.5	Normal	3	34	Follicles only
	8.7	4.5	Normal	6	59	Follicles only
FSH 96	3.4	4.5	Normal	6	30	Follicles only
	6.8	4.5	Normal	6	41	Follicles only
FSH 149	4.4	4.5	Normal	3	67	Follicles only
	8.8	4.5	Normal	3	84	Follicles only
FSH 4	3.2	4.5	Normal	3	41	Follicles only
	9.6	10.0	Hypophysectomized	6	130.5	Follicles only
	19.2	10.0	Hypophysectomized	8	192	Follicles only
FSH 126	11.2	4.5	Normal	6	29	Follicles only
FSH 535	8.1	4.5	Normal	6	34	Follicles only
F 116 A	0.67	4.5	Normal	19	81	Follicles few corpora
F 116 B	0.76	4.5	Normal	9	81	Follicles few corpora

RESULTS

Effect of *In Vitro* Addition of FSH.

Data obtained show (Table 2) that the addition of FSH to the medium in which chick ovary slices are suspended causes an increase in the rate of oxygen uptake by the tissue. This increase was demonstrated in three different media, being particularly marked in the

[3] The hormone preparations and their bioassay values were supplied by Dr. W. H McShan of the Department of Zoology, University of Wisconsin.

medium containing pyruvate. Comparison of "Control" values shows that addition of pyruvate to the buffered saline solution without hormone causes an increase of approximately 35% in QO_2 while comparison of "Test" values shows that the addition of pyruvate to the buffered saline solution containing FSH causes an increase of 60 to 80% in QO_2. If the increased rate of oxygen consumption in the two cases can be regarded as a measure of oxidation of pyruvate it appears that FSH causes an increased rate of oxidation of this substance by ovarian tissue. Direct measurements of the utilization of pyruvate by ovarian tissue are being made in order to substantiate this conclusion. Addition of glucose to the medium without FSH has little effect on the QO_2 but with FSH present there is a definite increase on addition of glucose (Table 2).

The FSH preparations used in this study were not chemically pure. The sample used in the experiments reported in Table 2 was biologi-

TABLE 2. EFFECT OF ADDITION OF FSH IN VITRO ON RATE OF O_2 CONSUMPTION BY CHICK OVARY SLICES

FSH 5367 Amount of FSH added to flask (mg.)	QO_2 (Averages and standard deviations of 3 to 7 determinations)								
	Medium: Krebs-Ringer phosphate solution			Medium: 0.02 M. Glucose in Krebs-Ringer phosphate soln.			Medium: 0.018 M. Pyruvate in Krebs-Ringer phosphate soln.		
	Control	Test	% change	Control	Test	% change	Control	Test	% change
1	6.6±0.6	7.2±0.7	+ 9				8.4±0.4	11.5±0.4	+37
2	7.0±0.4	7.9±1.1	+13*	7.2±0.3	8.9±0.8	+24			
3				7.4±0.2	9.5±0.9	+28	8.3±0.4	12.6±0.1	+52
4	7.0±0.4	8.3±0.3	+19	7.2±0.3	9.3±0.9	+29	8.3±0.4	13.2±0.2	+60
5	6.6±0.6	7.7±0.9	+17	7.2±0.3	10.0±0.9	+39	8.4±0.4	14.0±0.8	+67
20	6.3±0.3	8.7±0.6	+38				8.4±0.4	14.7±0.8	+75

* Difference not statistically significant; all other differences between control and test in same medium significant ($p = .01$).

cally pure in the sense that it was free from other gonadotrophic hormones as judged by bioassay methods, but it was not a homogeneous substance. The question arises, then, whether the effects observed on addition of this substance to ovarian tissue are due to the hormone proper or to impurities present in the preparation. First of all, it was shown that the hormone preparation itself did not take up oxygen under the conditions of the experiments. FSH 5367 and FSH 534 were prepared from sheep pituitary glands by the method of McShan and Meyer (1940) in which FSH is separated from LH by digestion of the latter with trypsin. Substances which might be expected as impurities in such a preparation are products of tryptic digestion and possibly traces of trypsin itself. Of these only trypsin would be destroyed by boiling. The hormone being a protein would be expected to be denatured by boiling. Therefore, if a boiled solution fails to produce the effect that the same solution produces before boiling one can conclude that the effect is not due to the impurities which are unaffected by such treatment. In experiments to test the effect of a boiled preparation of FSH the hormone was dissolved in Krebs-Ringer phosphate

solution containing pyruvate and boiled for 20 miuutes under reflux in a water bath. For control measurements untreated FSH was added to an aliquot of the medium which had received similar treatment. Results are shown in Table 3. Boiling of the preparation lowered its effect upon the rate of oxygen uptake by chick ovary slices. In the experiments using 5 and 20 mg. doses of FSH the effect disappeared completely in the boiled preparations. In the other cases there was a distinct lowering of effect but some activity remained. This residual activity may have been due to impurities present or to incomplete inactivation of the hormone.

TABLE 3. EFFECT OF BOILED AND OF DIALYZED PREPARATIONS OF FSH ON QO_2 OF CHICK OVARY SLICES. EFFECT OF GONADOTROPHIC HORMONE SAMPLES PREPARED WITHOUT TRYPTIC DIGESTION

Hormone	Amount added (mg.)	QO_2 (Medium: 0.018 M. Pyruvate in Krebs-Ringer phosphate)			
		No hormone	"Untreated" hormone	Boiled hormone	Dialyzed hormone
FSH 5367	5	9.9	11.5	10.0	
FSH 5367	10	8.0	12.1	9.5	
FSH 5367	20	9.2	12.7	8.9	
FSH 534	10	8.4	13.1	9.7	
FSH 534	10	8.5	12.5	9.2	
FSH 534	10	8.6	14.4	9.1	
FSH 96	6	7.7*	12.1		11.2
F 116 A	10	7.5 ±0.5	8.9 ±0.2		
F 116 B	10	7.8 ±0.5*	10.2 ±0.8		

* Ovaries from cross-bred chicks used in these determinations.

Data with FSH 5367, FSH 534, and FSH 96 are from single determinations.

Data with F 116 A and F 116 B are from two determinations (in duplicate) with each preparation. The differences with F 116 A and F 116 B are significant ($p = .01$).

Dialysis of a solution of the FSH preparation should remove any inorganic contaminants as well as products of tryptic digestion so this treatment would destroy the effect of the preparation on ovarian tissue if that effect were due to any of these impurities. Fifty milligrams of FSH 96 were dissolved in 4 ml. of distilled water and the solution placed in a dialyzing bag. This was placed in approximately 200 ml. of distilled water in a beaker and kept in the refrigerator for 24 hours, the water in the beaker being changed frequently during this period. The volume of liquid in the bag increased to about 5 ml. Six tenths milliliter of this solution per flask was used in the experiment. Control flasks with no hormone received 0.6 ml. of distilled water and control flasks with hormone received 0.6 ml. of distilled water containing 6 mg. of untreated FSH 96. Volumes in all flasks were made up to 3 ml. with Krebs-Ringer phosphate solution containing pyruvate. The dialyzed preparation caused an increase in the QO_2 of the tissue which was not quite as great as that produced by the non-dialyzed preparation (Table 3). The lowering of effect may have been

due to loss of some FSH activity since the hormone had been in solution for 24 hours. Maddock and Heller (1947) suggest that inactivation of solutions of pituitary gonadotrophins stored at icebox temperatures may occur, for the most part, during the first few days of storage.

The experiments with boiled and with dialyzed preparations of FSH samples do not rule out the possibility that the effect of preparations of FSH on the rate of oxygen consumption of ovarian tissue may be due to traces of trypsin. In Table 3 are shown the effects of two samples of hormones which were prepared by a method not involving the use of trypsin, F 116 A and F 116 B. Both of these increased the QO_2 of chick ovary slices. From the results of the experiments reported in Table 3 it appears that the increase in QO_2 of ovarian tissue caused by addition of the FSH preparations is due to the hormone and not to impurities present in the preparations.

The possibility that the increase in QO_2 is a non-specific protein effect was tested by adding other proteins of pituitary origin to the system containing the tissue slices. The adrenocorticotrophic hormone and the luteinizing hormone were chosen for this purpose. Five determinations were made measuring the QO_2 of chick ovary slices in the pyruvate medium with no hormone and with 5 mg. doses of ACTH, LH, and FSH. Neither ACTH nor LH affected the rate of oxygen uptake by the tissue, the average QO_2 values being 7.9 with ACTH and 8.9 with LH as compared to 8.1 with no hormone. In contrast FSH caused a highly significant increase producing an average QO_2 value of 11.1 ($p = .01$). The slight though not significant increase obtained with LH may be suggestive since LH might be expected to have a trophic effect on the interstitial (androgen-producing) cells of the ovary.

An experiment was performed to determine whether the magnitude of the increase in QO_2 of chick ovary slices produced by FSH preparations *in vitro* is correlated with the gonadotrophic potency of the preparations as judged by the increase in weight of ovaries of immature rats caused by injection of these preparations. By such bioassay FSH 4 was found to be considerably more active than FSH 535 and FSH 126. The latter two preparations were not greatly different in activity though FSH 535 was somewhat more potent (see Table 1). These three preparations were tested for their effect upon the QO_2 of chick ovary slices in the pyruvate medium. Five milligram doses of the hormones were used. The average QO_2 values of three determinations were: no hormone, 8.3; FSH 4, 10.3; FSH 126, 9.9; FSH 535, 8.4. The increases produced by FSH 4 and FSH 126 are significant but they are not significantly different from each other. FSH 535 in a 5 mg. dose did not produce a significant increase in QO_2 of the ovary slices. Thus, no correlation between gonadotrophic potency

as determined by bioassay and the magnitude of the effect on QO_2 of chick ovary slices was demonstrated by this experiment.

In Fig. 1 are plotted the data from Table 2 showing the effect on QO_2 of chick ovarian tissue of varying the concentration of FSH in the medium in which ovary slices are suspended. The concentration of the hormone within the cells of the tissue is probably not identical with the concentration in the medium but rather a function of it. It can be seen that the effect on QO_2 increases with concentration of the hormone up to a certain level and that the concentration which produces the maximum effect is lower in the medium containing

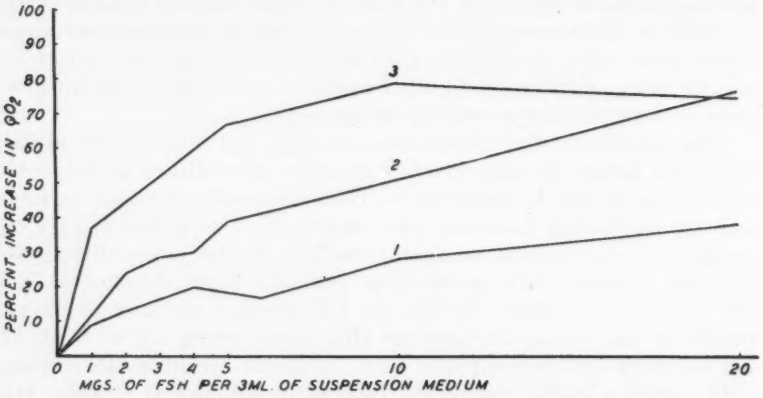

FIG. 1. Effect of Varying Amounts of FSH Added *in Vitro* on the QO_2 of Chick Ovary Slices.

1. Tissue suspended in Krebs-Ringer phosphate solution
2. Tissue suspended in Krebs-Ringer phosphate solution plus glucose
3. Tissue suspended in Krebs-Ringer phosphate solution plus pyruvate

pyruvate than in the other media. With glucose the maximum effect may be reached at a lower concentration than is indicated on the curve since no measurements were made at the 10 mg. level. The point at the 20 mg. level is based on only two observations (not included in Table 2). The points at the 10 mg. level on the other two curves are also based on two observations in each case.

In Table 4 are shown the results of experiments in which the rate of oxygen consumption of ovary slices from chicks that had received injections of FSH was compared with that of ovary slices from untreated control chicks. In the first two experiments test animals received subcutaneous injections of FSH daily for 10 days and were killed on the eleventh day for the respiration measurements. There was no significant difference between respiratory rates of the ovaries of these birds and those of untreated controls. Since exogenous FSH is probably rapidly inactivated by the liver it seemed likely that the 24-hour interval between the time of the last injection and the time of

killing allowed ample time for the FSH concentration of the blood and the ovaries of the treated animals to return to the endogenous level. So a third experiment was performed in which the test animals were given a large dose of FSH in a single intravenous injection and were killed 5 to 15 minutes later. In these the FSH level in the ovary should have been higher than that of the untreated controls. The QO_2 measurements were made in the pyruvate medium only. There was found to be an increase due to the injection of FSH which though small was highly significant ($p = .01$).

TABLE 4. EFFECT OF INJECTION OF FSH INTO CHICKS ON RATE OF O_2 CONSUMPTION BY OVARY SLICES

FSH	Total dose (mg.)	Injection period and site	Time lapse between last injection and killing of animal	QO_2 (Averages and standard deviations of 4 to 5 determinations)					
				Control			Test		
				Krebs-Ringer phosphate	0.02 M. Glucose in KRP	0.018 M. Pyruvate in KRP	Krebs-Ringer phosphate	0.02 M. Glucose in KRP	0.018 M. Pyruvate in KRP
96	34	Daily for 10 days S.C.	24 hrs.	6.8±0.4	7.1±0.5	8.2±0.2	6.8±0.4	7.3±0.4	8.7±0.5*
149	26	Daily for 10 days S.C.	24 hrs.	6.2±0.3	6.7±0.6	8.3±0.5	6.6±0.5	6.6±0.3	8.6±0.6*
96	100	Single Injection I.V.	5 to 15 min.			8.0±0.7			9.0±0.6†

* Differences between comparable QO_2 values of control ovaries and test ovaries not significant.
† Difference between QO_2 of control ovaries and test ovaries significant ($p = .01$).

DISCUSSION

From the results of the experiments reported here it appears that FSH affects some enzyme system or systems involved in oxidative mechanisms in ovarian tissue. Whether the effect is produced by direct participation in such a system, by release of some physiological inhibitor of it, or by some other mode of activation cannot be said, but the magnitude of the effect seems to depend upon the concentration of the hormone present in the tissue. This is apparent both from results of experiments varying the amount of FSH added to the medium in which ovary slices are suspended and the results of the experiments in which test animals received FSH by injection. The latter experiments indicate that the presence of the hormone in the tissue at a given time is necessary for its effect on oxygen consumption, that there is not an initial stimulation by FSH which is sustained after the hormone level drops.

In a review of the literature on the development of the Graffian follicle Hisaw (1947) points out that follicular growth can proceed in mammals to the stage of antrum formation in the absence of the pituitary gland and that it is only as the animal approaches sexual maturity that its ovaries become responsive to pituitary gonadotrophins, while the ovaries of the very young animal are refractory. Furthermore, quoting from the same review, "it is also a common observation that even in the adult animal all the follicles are not equally

responsive. When a pituitary extract is given in small doses to a rat the effects may be limited almost entirely to the larger follicles, but if the dosage is increased smaller follicles are called into the reaction, while the primordial follicles fail to be stimulated even when large amounts of the preparation are injected." There is a long period in the chick (150 days) in which the ovary does not respond to administered mammalian FSH with increase in weight of the organ as the criterion of response (Nalbandov and Card, 1946). It is interesting that the ovaries in which the increase in QO_2 due to FSH was observed were from chicks of this "refractory" age. Because of this observation it seems possible that the initial effect of FSH (and possibly the only effect) is the activation of oxidative mechanisms in the ovary thereby making an increased amount of energy available to the organ for developmental processes and that the lack of the end response, growth, in the ovary of the very young animal is due not to lack of response to the hormone but to some limiting factor in the subsequent chain of events leading to growth of the organ. Is it not possible that the mechanism for development of the ovary to full maturity is present in the organ itself and is controlled by the pituitary hormones in only a quantitative way by regulation of the energy available for its function? Such a concept would explain the lack of response to administered FSH of the ovary of the very young animal if we assume an orderly pattern of development of the organ regulated by endogenous mechanisms for which certain quanta of energy are required. Increasing the available energy before it is needed would produce no change in the pattern. On the other hand, lack of the pituitary hormones when the organ has reached the stage where a greater amount of energy is required would disrupt the pattern.

SUMMARY

The addition of FSH to the medium in which chick ovary slices are suspended causes an increase in the rate of oxygen uptake by the tissue. The magnitude of the response to FSH *in vitro* depends upon the concentration of the hormone in the medium as well as upon the substrate added to the medium.

The effect of FSH on the QO_2 of chick ovary slices is destroyed or lowered markedly in boiled preparations of the hormone, but is lowered only slightly in a dialyzed preparation, indicating that the effect is produced by the hormone rather than by impurities in the preparation. That the effect is not due to a non-specific protein action is shown by the fact that neither ACTH nor LH added to the suspension medium affects the rate of oxygen consumption of chick ovary slices.

The QO_2 of slices of ovaries from chicks that had received daily subcutaneous injections of FSH with a 24-hour lapse of time between the last injection and measurement of the respiratory rate of the ovaries was not significantly different from that of ovaries of un-

treated control animals. A single intravenous injection of FSH with an interval of 5 to 15 minutes between the time of injection and that of killing of animals for manometric measurements caused a significant increase in the QO_2 of the ovary in a medium containing pyruvate as compared to the QO_2 in the same medium of ovaries of untreated control chicks.

The possible significance of these findings with respect to the mechanism of action of FSH is discussed.

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NOTES AND COMMENTS

FAILURE OF ADRENOCORTICAL EXTRACT TO MODIFY THE IMMUNITY ACQUIRED BY INTACT MICE THROUGH THE USE OF PNEUMOCOCCAL VACCINE[1]

The low resistance of Addisonian patients and adrenalectomized animals to infection has led to speculation that adrenal function may influence the processes concerned with acquired immunity (Perla and Marmorsten, 1941). In support of this view two groups of investigators (Fox and Whitehead, 1936; Chase, White and Dougherty, 1946) have presented data indicating increased antibody formation in intact animals treated simultaneously with antigens and adrenocortical extracts. Since these results were not confirmed by other investigators (Eisen, Mayer, Moore, Tarr and Stoerck, 1947; Murphy and Sturm, 1947; Thatcher, Houghton and Zeigler, 1948) it was thought that another approach, independent of *in vitro* antibody determinations, might yield pertinent information.

METHODS AND PROCEDURES

The method chosen was to test the resistance of mice to active pneumococcal infection, after treatment with adrenocortical extracts and appropriate antigens, upon the assumption that any general increase in antibody would include an increase in specific protective substances. White Swiss mice weighing 18–22 grams were immunized with intraperitoneal injections of heat-killed Type I Pneumococci, and challenged 4–14 days later with living cultures of the same organism. Undiluted broth cultures contained approximately two billion diplococci per cc.; the bacteriological procedures have been described elsewhere (Vollmer and Samsell, 1949). Adrenocortical extract (ACE)[2] was administered subcutaneously in unitary doses of 0.5 cc., a quantity reported to produce lymphopenia in mice (Dougherty and White, 1944) or of 0.2 cc., which in repeated doses with antigens has been reported to enhance the antibody titer (Chase, White and Dougherty, 1946). As a control for aqueous ACE, which contained 10 per cent ethanol, isotonic saline solution containing 10 per cent ethanol was used.

RESULTS

Treatment of previously immunized mice with ACE given shortly before infection: Preliminary trials had shown that when a group of mice had been immunized with a given dose of killed pneumococci, 60–90 per cent of them would die after injection with the same dose of living pneumococci ("equivalent dosage"). This relationship was utilized in an attempt to determine

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[1] The authors assume full responsibility for the statements made in this report, which in no way reflects official naval opinion.

[2] Adrenal Cortex Extract (Upjohn), equivalent to 0.2 mg. 11-dehydro-17 hydroxycorticosterone per cc.

whether the injection of ACE in immunized mice just before infection would alter their mortality rate. One hundred twenty mice (60 males, 60 females) were injected with 0.05 cc. of a 10^{-3} dilution of standard vaccine. Two weeks later they were separated into two equal groups and injected with 0.5 cc. of ACE or saline. One-half hour later all were challenged with 0.5 cc. of a 10^{-4} dilution of a viable culture (equivalent dose). Only three controls and five hormone-treated mice survived.

Attempt to increase protection with one or two treatments of ACE at time of immunization: In a parallel experiment 120 mice were immunized with killed pneumococci and challenged four days later. In this instance the extract or

TABLE 1. SURVIVAL OF MICE IMMUNIZED WITH SIX INJECTIONS OF VACCINE WITH SIMULTANEOUS INJECTIONS OF ADRENOCORTICAL EXTRACT OR CONTROL SOLUTIONS, AND CHALLENGED WITH LIVING PNEUMOCOCCI

Vaccine dilution (0.05 cc.)		Number of survivors per group of six mice, challenged with Inocula (0.5 cc.) in series of three-fold dilutions, beginning with undiluted broth culture (A)										
		A	B	C	D	E	F	G	H	I	J	Survivors
10^{-2}												
	Exp.	0	0	0	1	4	4	6	6	6	6	33/60
	Con.	0	0	0	2	3	5	5	6	5	6	32/60
10^{-3}												
	Exp.	0	0	0	0	0	1	1	2	5	6	15/60
	Con.	0*	0	0	0	0	2	1	4	3	6	16/59
Survivors												
	Exp.	0	0	0	1	4	5	7	8	11	12	48/120
	Con.	0	0	0	2	3	7	6	10	8	12	48/119

* Five mice in this group.

control solution was administered at the same time as the immunizing vaccine. Again, the difference between the terminal mortality rates was very small, 20 controls and 22 treated animals surviving the injection.

Two similar experiments, with the conditions altered slightly, were carried out with similar results. In the first, a merthiolate-preserved adrenocortical extract[3] was administered at the same time as the immunizing vaccine. In the second, two doses of the alcohol-preserved extract were administered, one an hour before the immunizing vaccine and the other three hours later. Upon challenge with live pneumococci 63 (of 84) and 56 (of 90) mice died in the extract-treated groups, and 68 (of 84) and 51 (of 90) died respectively in the control groups.

Treatment with ACE at time of immunization, with varied protective and challenging doses: Each of 84 mice was given 0.5 cc. of ACE and simultaneously inoculated with one of four ten-fold dilutions of vaccine. They were later challenged, along with 84 controls, with inocula of bacteria of five different dilutions. The treated and control animals had the same pooled mortality rate (47/84) and similar mortality rates in their corresponding subgroups.

Repeated treatment with ACE and vaccine, simultaneously administered:

[3] Adrenal Cortex Extract (Wilson), equivalent to 75 gm. of fresh adrenocortical tissue per cc. This extract contains sodium ethyl mercuric thiosalicylate (1/100,000) and the control mice were therefore treated with isotonic saline containing the same amount of this preservative.

120 male mice were treated with 0.2 cc. of ACE at the time of each of six successive doses of vaccine (two weekly for three weeks), and 120 controls were treated with 0.2 cc. of saline solution and the vaccine. One week after the last treatment the mice were challenged with inocula of pneumococci, in a series of threefold dilutions. The pooled mortality rates of the experimental groups and their controls were practically identical, and the distribution of deaths was essentially the same throughout the sub-groups (table 1).

COMMENT AND CONCLUSIONS

It was evident throughout that the degree of immunity acquired by groups of mice varied according to the immunizing and challenging doses of bacteria administered, but was not materially affected by their treatment with ACE. This finding is consistent with that of Eisen and colleagues (Eisen, Mayer, Moore, Tarr and Stoerck, 1947) that the use of ACE did not increase the formation of precipitins to type I pneumococcal polysaccharide in adrenalectomized rats, and tends to discount the possibility raised by the work of Chase (Chase, White and Dougherty, 1946) that in one of the adrenocortical substances a nonspecific physiological adjuvant to immune response might have been found. However, the endocrine and bacteriological variables of this field have not been adequately studied for the presence of modifying factors. Therefore while the present experiments appear to indicate that whole ACE may not stimulate any extra reaction to antigens in intact animals, they should not be interpreted to exclude the possibility of indirect effects upon the immunity response of animals under the stress imposed by active infection or by different experimental conditions.

SUMMARY

In order to ascertain whether or not adrenocortical hormones increase the immune response of intact animals to antigens, white Swiss mice were immunized with Type I pneumococcal vaccine and simultaneously treated with ACE or control injections. They were later challenged with living pneumococci of the same strain. Dosages of vaccine and challenging inocula were varied over a wide range. In all instances the results were negative, indicating that whole adrenocortical extract administered early in the immunization period did not modify the resistance of the animals to active homologous infection.

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GROWTH HORMONE AND FAT METABOLISM

IN A FURTHER STUDY of the possible identity of urinary adipokinin (Weil & Stetten, 1947) and a pituitary factor, the effect of purified growth hormone upon the fat content of the mouse liver has been studied. Purified growth hormone[1] was injected hypodermically into fasting mice and the fat content of their livers determined by methods previously described. In confirmation of observations of others (Szego & White, 1948, 1949), a striking increase in total liver lipids was found to follow the administration of as little as 100 μg. of hormone (Table 1), a doubling of the control value occurring after 7 hours.

TABLE 1. FEMALE MICE, FASTED FOR 7 HOURS, INJECTED SUBCUTANEOUSLY WITH 100 G GROWTH HORMONE PREPARATION, 3PKR3 ARMOUR & CO., IN 0.5 CC SALINE, 2, 4, AND 7 HOURS BEFORE SACRIFICE. CONTROLS INJECTED WITH SALINE 7 HOURS BEFORE SACRIFICE

Time interval	Number of mice	Weight of mice	Mean liver weight	Mean liver fat per 100 g. body weight
2 hours	16	21,5–24	1,61	413 ± 19*
4 hours	12	22–24	1,88	564 ± 13
7 hours	16	21,5–24	1,91	685 ± 15
controls	30	20–26	1,66	326 ± 11

* Standard error: $\sqrt{\frac{\Sigma(v^2)}{n(n-1)}}$

In an analysis of the mechanism of action of growth hormone, it is of interest to establish the chonological sequence of events which follows its administration. In this regard we consider it worthy of note that a statistically significant increase in the quantity of liver fat is seen within 2 hours of the administration of this hormone. Thus the adipokinetic action of growth hormone appears to occur simultaneously with its effect upon the level of blood amino acids which has been demonstrated 1 to 2 hours after administration of this hormone to rats (Russell & Capiello, 1949).

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[1] The purified growth hormone was supplied by Armour & Co., Chicago, through the courtesy of Dr. P. Munson and Dr. I. M. Bunding.

GOITEROUS CHICKS FROM IODINE-INJECTED EGGS[1]

WOLFF AND CHAIKOFF (1948 a, b, c) have shown that administration of large amounts of inorganic iodide inhibits synthesis of thyroxine in the normal thyroid gland. However, this inhibition is temporary unless nephrectomized animals are used to maintain the high blood level of inorganic iodine. It would appear that the developing chick embryo might also be an excellent subject for study of the effects of prolonged thyroid inhibition. The constant supply of iodine provided by injecting inorganic iodine into the white of fertile eggs should be somewhat comparable in effect to a single dose administered to a nephrectomized animal.

Prolonged inhibition of thyroid activity would be expected to result in enlargement of the gland as a result of increased secretion of thyrotropin by the pituitary. It is speculated that such an effect might be involved in the production of goiters in chicks hatched from eggs laid by hens fed diets containing synthetic thyroprotein (STP), especially since STP contains from 1.5–2.0 per cent inorganic iodine as iodide (Graham 1948). The present communication reports a preliminary test of this hypothesis.

EXPERIMENTAL

Logarithmic doses of inorganic iodine as KI in sterile distilled water were injected into the white of fertile eggs on the 16th day of incubation. On the day of hatch the chicks were exsanguinated by decapitation and the thyroids were immediately excised and weighed on a Roller-Smith balance.

Levels of inorganic iodide from 156μ to 40,000μ per egg resulted in progressive and significant thyroid enlargement; dosages from 0.6μ to 39μ failed to produce goiter (Table 1). At the dosage levels which produced goiters incubation time was increased and the navels of the chicks were incompletely closed. Embryonic mortality was apparently greater at the higher levels but the numbers in each group are not sufficiently large to justify a positive statement.

TABLE 1. GOITEROGENIC INFLUENCE OF INORGANIC IODIDE (AS KI) WHEN INJECTED INTO FERTILE EGGS ON THE 16TH DAY OF INCUBATION

Amt. I inj. in each egg	No. chicks hatched[1]	Mean thyroid weight at hatch	Ratio to control
(gamma)		(mg ± SE)	
40,000	3	10.0 ± 0.34	3.23
10,000	4	9.6 ± 0.82	3.10
2,500	8	8.9 ± 0.58	2.90
625	9	7.3 ± 0.52	2.35
156	8	5.8 ± 0.52	1.90
39	7	4.3 ± 0.46	1.40
9.8	6	3.8 ± 0.38	1.20
2.5	9	4.4 ± 0.38	1.40
0.6	9	3.8 ± 0.30	1.20
Control	9	3.1 ± 0.13	—

[1] Ten fertile eggs were injected with each dosage, but not all the chicks hatched.

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[1] This study was made possible through funds made available jointly by the Carnegie Foundation and the University of Georgia. The writers, however, are solely responsible for the statements made in this report.

COMMENT

In earlier studies the present writers have shown a quantitative relationship between level of STP fed to the hen and increase in incubation time and degree of thyroid enlargement of their chicks (Wheeler and Hoffmann, 1948 a, b). An hypothesis that this effect was due to reduced maternal thyroid activity with an attending failure of the thyroidal substance to be deposited in the egg proved untenable when the feeding of desiccated thyroid to hens failed to alter incubation time or thyroid size of their chicks (unpublished data). Since the iodine content of the hen's diet determines the iodine content of the egg (Wilder, Bethke and Record, 1933), the work of Wolff and Chaikoff provides an alternative hypothesis in that the inorganic iodide present in the STP fed to hens might increase the iodine content of the egg sufficiently to inhibit thyroxin synthesis by the embryonic chick thyroid. This in turn would allow increased thyrotropin secretion and thyroid enlargement. Such a mechanism would adequately explain the goiters found in newly hatched chicks as well as their hypothyroid symptoms such as the lowered oxygen consumption rates observed by McCartney and Shaffner (1949) and poor closure of the navel.[2]

It may be appropriate to point out that although the injection of STP into eggs was comparable in its effect to the injection of an equivalent amount of thyroxine (Booker and Sturkie, 1949), the validity of the present data is in no way impaired because the simultaneous injection of thyroxine with iodine (both of which are present in STP) would inhibit the release of thyrotropin by the pituitary.

The present findings show that injected inorganic iodide will produce goiter in chicks and increase incubation time. Work is now in progress to test whether the feeding of inorganic iodide to hens will also result in the production of goiterous chicks.

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[2] Poor closure of the navel has been established as a symptom of hypothyroidism in the progeny of pigeons made hypothyroid as a result of iodine deficiency (Hollander and Riddle, 1946), and in chicks from thiourea-injected eggs (Grossowicz, 1946).

[3] Our indebtedness to Mr. John Brooke for technical assistance is gratefully acknowledged.

ASSOCIATION NOTICE

ANNOUNCEMENT

The membership roll of the Association is being revised in preparation for the issuance of a new Roster as of December 31, 1949. Copy for this must be in the hands of the printer not later than October 1, 1949.

Return postal cards are being mailed to members requesting the following information:

Name.....Degrees.....

Mailing Address.....

.....

Teaching or Research Position:.....

.....

.....

If in active practice of medicine, specialty:.....

.....

Members are urged to complete and return the card immediately and to keep the Secretary informed at all times of any change in the above.

HENRY H. TURNER, M.D.
Secretary-Treasurer
1200 North Walker Street
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